THE EFFECT OF INCREASED FUNCTIONAL FIBER CONSUMPTION ON MICROBIOTA COMPOSITION IN INDIVIDUALS WITH CHRONIC KIDNEY DISEASE

By

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To Alexis

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I thank my parents, family, friends, and mentors. They have inspired and encouraged me in all areas of my life. I will continue to use the skills that they have taught me in all of my future endeavors.

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Twenty six million American adults suffer from chronic kidney disease (CKD) and millions of others are at risk of developing the condition. Many of the symptoms associated with CKD are a direct result of the accumulation of the byproducts of normal digestive and metabolic processes that occur in the body. In healthy individuals metabolic wastes, such as urea from protein metabolism, are filtered from the blood by the kidney and excreted in the urine. Individuals with CKD have insufficient filtering capacity, leading to the accumulation of waste products in the blood and the accompanying perturbations in body functions. Those toxins that interfere with other metabolic processes are collectively known as uremic retention molecules (URMs). The current dietary method of treating CKD involves consumption of a diet moderate in protein. Protein restriction has some efficacy in reducing the production of nitrogenous wastes, and lowering blood urea nitrogen (BUN) levels, but it may also contribute to malnutrition, and further the general decline in health associated with CKD. Other therapeutic targets for individuals with CKD should therefore be considered. The gut

microbiota may be one such target, as microbial metabolism may modulate blood levels of URMs through fermentation of undigested materials in the colon.

The aim of this study was to determine the effects of adding foods fortified with 23 g/d of functional fiber to the diets of patients with chronic kidney disease. Specifically, our goal was to determine the effect of the fiber on the composition of the gut microbiota of these individuals. Adults (n=14) with a mean age of 64.8$\pm$14.7 were recruited and enrolled in a 6-week single–blind, crossover design study. Study participants consumed commercially-available food products containing no added fiber for a 2-week control period, followed by a 4-week intervention period in which they consumed similar foods with 23 g of added fiber. Stool samples for microbiota analysis were collected during the second and sixth weeks of the study. Compliance with food intake was 83% for control, and 78% for treatment. Consuming foods with added fiber had no significant effect on microbial diversity, structure of the microbiota, or the numbers of lactic acid bacteria or bifidobacteria, but did appear to cause changes in the proportion of individuals containing specific operational taxonomical units within the microbiota. In conclusion, high functional fiber intake in individuals with chronic kidney disease causes changes in the gut microbiota, however, the physiological effects of these changes are not known.

CHAPTER 1
LITERATURE REVIEW

Kidney Structure and Function

The kidneys are a pair of bean-shaped organs located near the middle of the back, just below the ribcage, in the retroperitoneum [1]. They have a number of vital functions in mammalian species. Most important is their role in maintaining homeostasis by regulating fluid and electrolyte balance. The kidneys regulate blood volume, pressure, osmolarity, and pH by filtering the blood and selectively excreting or reabsorbing fluid and solutes as they pass through the renal tubules [1-3]. This absorption and excretion is regulated by the hormones aldosterone, antidiuretic hormone, and parathyroid hormone [1]. These hormones act on various structures within the intricate filtration and gradient systems within the kidneys and are released as a result of changes in body such as blood osmolarity, pressure, and pH [1]. This system is essential for the efficient elimination of materials that are produced during the normal breakdown of nutrients and body tissues.

The kidneys are composed of three distinct regions, the renal cortex, renal medulla, and renal pelvis [1]. Each of these regions is structurally distinct and houses a different step of the filtration process. The renal cortex is the outermost layer of the kidney, located between the renal capsule and the medulla [4]. The renal cortex is the site where blood filtration begins and is the location of the blood filtration machinery. The next layer is the medulla, located just under the renal cortex. The renal medulla is responsible for maintaining the osmolarity of the blood [4]. Because the fluid in the renal medulla is hypertonic to the filtrate, it is here that fluid moves out of the tubule and

reenters circulation. The renal pelvis is the innermost region of the kidney where urine collects before moving to the bladder [4].

Production of urine requires several complex steps that occur within units called nephrons [2, 4].The nephrons are the functional unit of the kidney and its various structures run through the three regions of the kidney. There are roughly 1 million nephrons in each kidney and collectively they filter about 189 liters of blood every 24 hours [1]. Filtration of the blood begins in the cortex, where blood is filtered by the Bowman's capsule [4]. Blood from circulation enters a bundle of capillaries called the glomerulus via the renal artery [1]. Small molecules such as sodium, chloride, calcium, and potassium ions are pushed through the small fenestrations of the capillaries and out of circulation by blood pressure. These solutes then travel through the bowman's capsule, and become filtrate [1]. Large molecules, such as proteins, and cells cannot pass through the fenestrations of a healthy nephron and remain in circulation. Materials that the body needs, such as glucose, are reabsorbed as the filtrate passes through semi-permeable tubing that composes the renal tubule. Throughout the renal tubules, the filtrate's composition is changed dramatically. Molecules like sodium and potassium are removed from or added to the filtrate according to the body's needs [1, 4]. Water is also removed from the filtrate at specific locations by the actions of aldosterone and antidiuretic hormone. These hormones act by different mechanisms, but both function to conserve water in periods of dehydration or low blood volume [1].

Chronic Kidney Disease

Chronic kidney disease (CKD), or chronic renal disease, is defined as the gradual and permanent loss of renal function over months or years [1, 4]. There are a

number of potential conditions that can cause decline in kidney function. The most common are diabetes mellitus and hypertension [3]. Glomerulonephritis, inherited diseases, malformations, obstructions, and repeated urinary tract infections can also lead to kidney damage and CKD [1, 4]. These conditions may cause progressive and irreversible damage to the nephrons. As nephrons are damaged, the remaining functioning nephrons adapt to maintain adequate renal function. This adaptation involves accelerated filtering, a process called adaptive hyperfiltration. Initially this hyperfiltration allows individuals with CKD to maintain seemingly normal renal function, but ultimately leads to damage and loss of remaining nephrons [5]. Renal function is determined by the filtering capacity of the kidney, measured as the glomerular filtration rate (GFR). This rate is used to determine the progression of CKD and describes the rate of filtered fluid that moves through the glomeruli and into the bowman's each minute. The GFR can be calculated by measuring any chemical that has a steady level in the blood and is easily filtered, but not reabsorbed or secreted by the kidneys. The normal GFR range is similar in men and women and ranges from 100-130 ml/min/1.73m^2 for adults, this value is usually estimated using blood creatinine [6]. In children the GFR is calculated using injected inulin clearance. CKD is classified as having a GFR of less than 60 mL/min/1.73 m^2 for three months regardless of the presence or absence of kidney damage [7]. There are five stages of CKD marked by set ranges of GFR. Filtering capacity is diminished with each advancing stage with stage 5 being established renal failure. Stage 5 is also called end stage renal disease and is classified by a GFR of less than 15 mL/min/ 1.73 m^2, these patients do not have adequate renal capacity to maintain life and must therefore undergo dialysis and

potentially transplantation [6]. Due to hyperfiltration, and other adaptive mechanisms, the symptoms of CKD may be mild until the disease is in its later stages. It is reported that symptoms are usually observed when renal capacity is only one-tenth of normal [6]. Symptoms may include malaise, poor concentration, anorexia, edema, itching, polyuria, and trouble sleeping or concentrating. Individuals with CKD also have an increased risk of developing hypertension. Cardiovascular events associated with CKD include angina, myocardial infarction, heart failure, stroke, and sudden death [8]. The risk of these events increases with the progression of the disease. Longitudinal studies have shown that cardiovascular disease is the leading cause of death in elderly individuals with CKD [8, 9].

Uremia and Uremic Retention Molecules

As renal function declines, the accumulation of unfiltered metabolites in the blood and tissues increase causing dramatic changes in blood composition. These changes are accompanied by serious disturbances in body functions including disrupted electrolyte, mineral, and fluid balance as well as anemia, high blood pressure, acidosis, bone disease and other serious complications [8, 10]. These metabolites have various origins, but are collectively known as uremic retention molecules (URMs). URMs can be biochemically active, having an effect on biochemical and physiologic functions [10]. Uremia is defined as “the signs and symptoms accompanying CKD that cannot be explained by derangements in extracellular volume, inorganic ion concentrations, or lack of known renal synthetic products” [11]. It is characterized by the deterioration of biochemical and physiologic functions leading to a number of complex and variable symptoms. URMs that specifically disturb metabolic processes and lead to the uremic syndrome are known as uremic toxins and include urea, oxalic acid, parathyroid

hormone (PTH), phenols, and indoles [10, 11]. Uremic toxins affect nearly every organ system in the body, but are especially of concern because they contribute to the cardiovascular damage associated with CKD [10].

Each uremic toxin has a distinct origin, is present in different ratios, and has different effects throughout the body [12]. URM production can have exogenous, endogenous, or bacterial origins. Exogenous URMs come from the absorption of toxins already present in, or created as a byproduct of the digestion or metabolic modification of food products or drugs. Exogenous URMs can be foods or materials added to foods during processing such as preservatives, and flavor correctors, such as benzoic acid, and pennyroyal oil [13]. Endogenous URMs are those that are produced during metabolic processes, usually endocrine processes [7]. These endogenous URMs are created as a result of the breakdown of body tissues. The third, and often neglected source of URMs, are those that are created during the bacterial fermentation of undigested materials in the colon. These fermentation byproducts build up in the large intestine and are absorbed, contributing to the uremic condition [12, 14]. Changes in the intestinal flora or changes in the activity of the microbiota can therefore significantly contribute to increasing serum concentrations of many toxins.

There are a number of different URMs, but phenols, indoles, and amines are the principle compounds produced by microbial fermentation [11]. Phenolic compounds such as phenol, *p*-cresol, and phenylacetic acid result from the partial breakdown of the amino acids tyrosine and phenylalanine by *Lactobacillus, Enterobacter, Bifidobacterium, Clostridium*, and other obligate or facultative anaerobes. Phenolic compounds accumulate rapidly in the serum of uremic patients, even those undergoing dialysis [15].

Phenol and p-cresol are usually present in the highest amounts and are associated with uremic coma and gastrointestinal bleeding at concentrations of about 100 μmol/L [15]. Recent data indicates that these phenolic compounds circulate in the body as conjugates such as p-cresol sulfate and p-cresol glucuronide and that concentrations of these molecules in circulations can be used as a predictor of mortality in patients at different stages of chronic kidney disease [10].This correlation with morbidity may be a result of the inflammatory properties of these compounds, which contribute to cardiovascular disease, infectious complications, and other uremic symptoms, especially those of the central nervous system [16].

Indoles are protein-bound compounds that result from microbial fermentation and metabolism of tryptophan. Many enteric microbes, such as *E. coli*, have enzymes called tryptophanases that catalyze the conversion of tryptophan to indoles. The indoles are absorbed, along with fluid by the colon, allowing them to enter circulation. Many indoles result from this fermentation, but the most studied is indoxysulfate, which is conjugated in the liver. Indoxysulfate is believed to cause inflammation, renal fibrosis, and continued loss of kidney function [13]. Studies have linked indoxysulfate to endothelial damage, inhibition of endothelial repair, and oxidative stress [17].

Dietary Therapies for Chronic Kidney Disease

The goals of dietary therapies for individuals with CKD are to diminish the accumulation of nitrogenous wastes, limit the metabolic disturbances characteristic of uremia, to prevent malnutrition, and to slow the progression of renal failure [7]. Protein energy malnutrition is characteristic of CKD and is associated with an increase in mortality, due to inflammation and cardiovascular death risk [18]. It is reported that 40%

of individuals with CKD are malnourished at the start of chronic hemodialysis treatment. Of these individuals, 20% to 60% will remain undernourished while on an established hemodialysis routine [7]. Inadequate synthesis of erythropoietin, blood loss, and an iron deficient diet also makes anemia common in CKD patients [19, 20]. Disturbances in protein and energy metabolism, hormone levels, loss of appetite, nausea, and vomiting associated with uremia also contribute to poor nutritional status in these individuals. CKD is therefore associated with negative changes in nutritional markers such as decreased serum proteins, decreased body mass, and decreased nutrient intake [21].

Despite the goals of dietary therapies, there is insufficient evidence to suggest that dietary protein restriction slows the progression of CKD [7]. In general, dietary therapies at all stages of CKD involve consumption of a diet low in phosphorous, potassium, calcium, certain vitamins, minerals, and require moderate protein restriction [22]. The moderate protein content of these diets effectively decreases the production of nitrogenous wastes, relieving the symptoms of uremia, but may also decrease health outcomes. Foods excluded from these diets include dairy products, dried beans, nuts, fruit, starchy vegetables, and whole grains, which contain vital nutrients and are important for maintaining health. Limiting these foods, in addition to factors such as anorexia caused by the uremic state, altered taste, and limited means or resources may encourage poor diet for CKD patients. The lack of consumption of adequate protein may also be a large contributor in the decline in health. Many CKD patients undergoing dialysis experience consistently low levels of serum proteins, due in part to loss of protein in the urine and through dialysis [9]. This may suggest that protein restriction may be hazardous in individuals with CKD. Individuals who are not properly instructed,

do not follow dietary recommendations, or that do not have the means to follow such stringent regimens while maintaining variety in their diets are especially at risk of developing protein energy malnutrition.

According to the World Health Organization, the daily recommended allowance for protein in adults is 0.8g/kg/d, the minimum daily requirement is 0.6g/kg/d, many CKD diets suggest an intake near only 0.3g/kg/d [23].In healthy individuals, such dramatic protein restriction would trigger mechanisms such as suppression of amino acid oxidation, suppression of protein degradation, and increased protein synthesis in order to maintain protein balance. Individuals with severe renal insufficiency may be unable to activate these adaptive responses due to inflammation, acidosis, and infection [23].

In addition to limiting nutrients, restriction of the consumption of dried beans, fruits, and vegetables in CKD diets may promote low fiber intake. Furthermore, because these high-fiber foods are also naturally low in fat, low fiber diets may encourage increased fat intake [24]. Fiber is the component of carbohydrate that cannot be digested by human enzymes in the small intestine. It provides structure to parts of plant cells and is therefore found mostly in fruits, vegetables, nuts, grains, and legumes [25]. Because it cannot be digested in the small intestine, it is not absorbed by the body and passes through the digestive system where it can have a number of effects. The Institute of Medicine (IOM) definition of dietary fiber includes dietary fiber and functional fiber [25]. The Adequate Intake (AI) for total fiber is 38 g/d for men and 25 g/d for women. Median intakes in the United States are significantly lower than this at only 15 g per day [26]. Currently, food guidelines recommend daily consumption of beans, peas, fruits, whole grains, and other foods with naturally occurring fiber [26].With strict

restrictions on their consumption of many of these good sources of fiber, it can be inferred that consumption of fiber in individuals with CKD is less than adequate.

Diets high in fiber are associated with lower serum cholesterol levels, lower risk of coronary heart disease, better glycemic control, reduced blood pressure, reduction in certain cancers, and greater weight control [24, 27]. In addition to these benefits, fiber may have additional properties that are specifically beneficial to individuals with CKD. Individuals with CKD are in an acute and chronic pro-inflammatory state that contributes to mortality and morbidity [28]. Studies analyzing data from the National Health and Nutrition Examination Survey III, have shown an association between high dietary fiber intake and significant decreases in inflammation, both in the general population and in individuals with CKD, measured by C-reactive protein levels [28]. The decreases in C-reactive protein levels were also significantly different between the groups with and without CKD, with the greatest drop being in the CKD group. These studies also showed an inverse relationship between fiber intake and mortality in individuals with CKD [28].

Fiber sources can be broadly grouped into one of two categories; dietary fiber, or functional fiber [25]. Dietary fibers are intact non-digestible carbohydrates and lignin that maintain the strength and structure of plants. These plant carbohydrates are most often the polysaccharides that comprise cell walls and the intercellular matrix of the plant [29]. Dietary fibers are indigestible carbohydrate found in foods such as fruits, vegetables and beans. Dietary fibers include lignin, resistant starch, cellulose, beta-glucans, hemicelluloses, pectins, gums, inulin and oligofructose, and associated plant materials [25]. Because they are found intact in their food sources, dietary fibers may be present

in low amounts in CKD diets. Functional fibers consist of non-digestible carbohydrates that have been isolated and that have beneficial effects in humans [25]. Because functional fibers are not necessarily associated with potassium and phosphate like dietary fibers, they may provide a safe alternative fiber source for individuals with CKD. These fibers are extracted from intact fiber sources using a variety of techniques or may be manufactured such as in the case of synthetic resistant starches and oligosaccharides [29]. The category of functional fibers is extensive and includes psyllium, fructooligosaccharides, polydextrose, resistant dextrins, and other isolated ir synthesized fibers.

Fiber can be further classified as soluble or insoluble, viscous or non-viscous, and fermentable or nonfermentable. Each of these fiber classifications exhibits different properties and physiological responses, but some are used interchangeably based on their use. The soluble versus insoluble distinction is an analytical one based on how readily a fiber disperses in water. Soluble fibers are dispersible in water, while insoluble fibers are not. Soluble fibers include beta-glucans, gums, psyllium, some pectins and some hemicelluloses, and can be found in the highest levels in oatmeal, lentils, dried peas, beans, and certain fruits and vegetables. Insoluble fibers have a laxative effect because they hold water and bulk the feces. Lignin, and some hemicelluloses are also insoluble fibers. Many soluble fibers are also viscous. Viscous fibers are categorized by their ability to form gels when they are mixed into water. These gels form a matrix that can trap materials in the digestive tract and help reduce cholesterol levels and prevent postprandial spikes in blood sugar by slowing their digestion and absorption [31, 32].

Fermentable fibers are those that can be fermented by the microbiota in the colon. These fibers are associated with a number of health benefits because their bacterial fermentation results in the formation of short chain fatty acids (SCFA). SCFAs (acetate, butyrate, and propionate) are used as an energy source for colonocytes, the epithelial cells that line the colon. Of the SCFAs produced by bacterial fermentation, butyrate is the preferred energy source for these cells. Butyrate has been shown to have beneficial effects in different colonic diseases and may be protective against colon cancer and diverticular disease [33]. Butyrate may exhibit these effects by increasing colonic blood flow, regulating colonic motility and by enhancing colonic healing [32, 34].

Of particular interest are soluble, fermentable fibers called prebiotics. A prebiotic is a nondigestible carbohydrate that beneficially affects the host by selectively stimulating the growth and, or activity of bacteria that are considered beneficial [27]. In order to be categorized as a prebiotic a food material cannot be broken down or absorbed in the upper digestive tract, it must selectively be fermented by beneficial bacteria, and it must therefore help create a more “healthy” bacterial profile by selectively enhancing the numbers of these bacteria inducing luminal or systemic effects that are beneficial to the host. [27] In most cases, nearly 90% of prebiotic fibers escapes digestion in the intestines and are fermented by bacteria in the colon [27, 35]. Prebiotic fibers such as the inulin, and oligofructose, are naturally found in a variety of plants, such as garlic, onions, artichoke, and chicory root. Most commercially-available inulin and oligofructose is synthesized from sucrose or extracted from chicory root by hot water extraction [27]. Oligofructose is obtained by treating inulin with hydrolytic enzymes to achieve partial hydrolysis[27]. Structurally these fructooligosaccharides are

short or medium length chains that cannot be cleaved by mammalian digestive enzymes [24].

Resistant starches are starches that make it all the way through the small intestine without being degraded by human digestive enzymes. The exact amount of starch that escapes digestion is variable, dependent on factors such as transit time, enzymatic activity, and the interaction of the starch with other materials in the digestive tract [27, 36]. Like fructooligosaccharides, resistant starches are associated with butyrate production, but to a greater degree. Resistant starches may also improve insulin sensitivity, promote satiety, lower cholesterol, increase beneficial bacterial species, and promote bowel regularity [36].

Resistant starches can be grouped into four categories RS1, RS2, RS3, and RS4. RS1 are resistant starches that are inaccessible or digestible, they are found in seeds, legumes, and whole grains that have not been processed [36]. RS2 resistant starch is granular starch that cannot be digestible by human enzymes. These starches can be found in unripe bananas, high amylose corn, and uncooked potatoes. RS3 starch is the result of cooking and cooling foods that contain resistant starches, such as rice, cornflakes, legumes, and potatoes. RS4 starches are created, or chemically modified to resist digestion [36]. RS_3 and RS_4 are not digested by mammalian intestinal enzymes are largely fermented in the colon. RS_1 and RS_2 are classified as Dietary Fibers, while RS_3 and RS_4 may be considered Functional Fibers [36].

The Colon and Gut Microflora

The large intestine has a number of important functions, with the principal function being reabsorption of fluid and electrolytes from the feces before defecation. In

addition, the colon also houses up to 10^{14} bacteria from hundreds of different species [37]. In healthy individuals a commensal relationship exists between the bacteria of the colon and the host [27, 37]. The host provides a suitable environment and fermentable substrate for these organisms and they in turn produce beneficial materials that can be absorbed and utilized by the host [37]. In reality, bacterial fermentation in the colon produces a wide range of materials that have both beneficial and harmful effects on the host. The balance between the harmful and beneficial compounds produced is determined by a number of factors and can be modulated by a number of different factors.

The large intestine is approximately 150 cm long and is divided into three functional regions called the cecum, colon and the rectum [38]. A steady flux of undigested material and the large number of niches create a suitable environment for a complex and very active community of bacteria. These organisms are mostly obligate anaerobes from the genera *Bacteroides, Fusobacterium, Eubacterium,* and *Bifidobacterium*, but others exist in high numbers as well [27, 39]. Colonization of the gastrointestinal tract begins during the birth process and over time the colonization pattern begins to resemble that of an adult [40, 41]. Factors that influence the bacterial species seen are the type of delivery, vaginal or via cesarean, the length of stay in the hospital after birth, whether or not the individual was breastfed and a number of other factors [40, 41].

Initially aerobes and facultative anaerobes predominate the colon, helping to produce a highly reducing environment that facilitates the growth of strict anaerobes [27, 39]. During vaginal deliveries infants are exposed to bacteria from the mother's

vagina, feces, and upper gastrointestinal tract [39-41]. This process allows a mother to transfer many of the bacteria from her own microbiota to her child. This can be positive in cases where the mother has a healthy flora, or detrimental in the converse case. Cesareans, use of antibiotics, and lengthy hospital stays are usually associated with pathogenic bacteria such as C. *dificile* [37, 39, 41]. The length of time that it takes for the gut microflora to stabilize can also be influenced by factors such as the type of delivery [40, 41]. The composition of the gut microflora differs significantly between individuals, even members of the same family [42].

A beneficial bacterial profile has been associated with the inhibition of the growth of harmful bacteria, improved digestion, synthesis of vitamins, and stimulation of immune functions [43-45]. Negative bacterial profiles are associated with diarrhea, infections carcinogenesis, and intestinal putrefaction [27, 37]. The composition and activities of the colonic bacteria are affected by physiological and structural factors such as substrate availability, pH, O_2 levels, and the position of the bacteria in the colon [46]. Physiological factors, such as pH and O_2 levels are largely determined by the presence of absence of other bacteria in the colon. Though the microbiota remains relatively fixed throughout the lifetime of an individual, slight changes in the structure of the microbiota can have significant affects [37]. Wang and Gibson (1993) showed that when bifidobacteria grown on fructooligosaccharide mediums, they reduce the numbers of bacteroides, clostridia, or coliforms [47]. This is thought to be due to a change in the pH, which is unfavorable for these pathogenic bacteria and, potentially by the production of bactericidal substances [47]. This interrelationship between bacteria is why antibiotic use is often associated with diarrhea and secondary infections [48]. Elimination of

beneficial bacterial allows for the growth of pathogenic species that lead to illness. Additionally, the bacteria that make up the microflora have the ability to adapt to conditions in the colon by changing metabolism, utilizing enzymes that allow them to metabolize available substrates and outcompete other bacteria [11].

Microbial Activity

The bacterial species that comprise the gut microflora are diverse and have the ability to change their activity based on a number of environmental conditions, especially nutrient availability. Saccharolytic bacterial species preferentially ferment carbohydrates; proteolytic bacterial species are primarily fermenters of protein [27, 49]. The distinction between the two groups is a result of structures or enzymes present in one group that are not present in the other. In the colon, the available substrate can be quite large. Fibers, lignins, and other indigestible materials generally make it to the colon intact. Specialized structures outside bacterial cells that house enzymes allowing them to digest cellulose and other polysaccharides extracellularly, provide an advantage. These bacteria can then take in the smaller sugars that result and use them for their metabolism, the byproducts of their digestion can also be used by other bacteria. This can also be the case with proteolytic enzymes, though proteins generally make it to the colon less intact than carbohydrates because they are degraded and denatured by mammalian peptidases.

Availability of nutrients appears to be the principle factor in determining the modulation of the metabolism of the microbiota [27]. Aside from colonic mucus, fiber is the primary carbohydrate source in the colon because it is resistant to digestion by mammalian enzymes [49]. Bacteria in the colon are able to use this carbohydrate as an

energy source, producing hydrogen, methane, and short chain fatty acids [50]. Short chain fatty acids are considered to be beneficial to the host, and have been proven to help maintain the integrity of the colonocytes [51]. Under these conditions, nitrogen sources found in the colon are used for growth and proliferation. In the absence of sufficient carbohydrate, organisms in the colon will begin to ferment protein as an energy source. The fermentation of protein is called putrefaction, and the process requires the hydrolysis of polypeptides into small oligopeptides and free amino acids that are then fermented and used for bacterial [11]. Many of the products of bacterial fermentation of proteins are toxic such as ammonia, amines, thiols, phenols, and indoles [11]. These materials can build up in the colon and be absorbed by the host, contributing to uremia in individuals with diminished kidney function [13].

CKD and associated changes in dietary habits influence several of the determinants of bacterial fermentation. Despite a reduction in protein intake, the accompanying reduction in fiber intake leads to a low carbohydrate to protein ratio [11]. This shift is associated with increased putrefaction, increased transit time, and a greater numbers of pathogenic bacteria in the microbiota [11]. All of these changes lead to more putrefaction, and the increased production of toxins. Recent research has shown that increasing intake of isolated fibers in individuals with chronic kidney disease can increase the fecal excretion of nitrogen relative to urinary excretion [35, 52]. This suggests that nitrogen is being cleared from the blood and shunted into the feces by increased bacterial incorporation. As fiber consumption increases, so does the proliferation of the intestinal bacteria that are able to utilize these materials, increasing the need for other compounds necessary for growth, such as nitrogen [51]. This

nitrogen is used in the formation of new bacterial proteins. The acidic environment of the colon created by short chain fatty acid production may also cause the conversion of ammonia into ammonium, which is less readily reabsorbed by the body. Limitations to this research are that the activity of the gut microflora changes significantly in different regions of the intestines, specifically between the proximal colon, where substrate is high, and the distal colon. The sensitivity of the structure and activity of the microbiota to the availability of substrate may provide an important target in developing new, safer therapies for individuals suffering from chronic kidney disease.

Molecular Techniques

One of the greatest limitations to the study of the microbiota is that many bacteria that colonize the gut cannot be cultured by conventional methods. Selective enrichment cultures fail to provide adequate conditions for these bacteria to grow and proliferate. For some time this made rare, slow growing, or uncultivable bacteria difficult or impossible to identify and characterize, providing an incomplete picture of the intestinal microbiota. Molecular techniques such as polymerase chain reaction (PCR) and high-throughput DNA sequencing have allowed the use of 16S rRNA sequences as an effective means of identifying and classifying new bacterial species [53]. The 16S ribosomal RNA of prokaryotes is about 1,500 nucleotides in length and is encoded by genes called 16S rDNA.16S rDNA has highly conserved sites, which can be used for primer binding and targeted amplification of these regions in all species present within a sample using polymerase-chain reaction (PCR) [53, 54]. Conserved sites flank highly variable regions, V1-V9, that appear to be highly similar within related organisms, specifically those of the same genus and species [54, 55]. Specific hypervariable regions are more suited for differentiating between particular types of bacteria. For

example, Chalravorty *et al.* (2007) discovered that the V6 region is able to differentiate between most bacteria, except enterobacteriacea, while the V1 best differentiates between *Staphylococcus aureus* and coagulase negative *staphylococcus* spp. [55]. Hypervariable regions between different bacteria can therefore be compared to one another as well as to reference sequences for identification and analysis of their relatedness [53, 54]. A great deal of information can be gleaned using PCR followed by sequence analysis, however, only qualitative information about the microbiota can be obtained using these techniques and species that are present in low numbers are not easily detected [53, 54].

Within the last few decades' techniques such as Denaturing gradient gel electrophoresis (DGGE) and real-time quantitative polymerase chain reaction (qPCR) have been used to qualify and quantify differences in microbiota profiles between different individuals or populations. Denaturing Gradient Gel Electrophoresis is a molecular technique that can be used to quickly compare microbiota composition between many individuals [56]. This comparison is based on changes in the electrophoretic mobility of 16s DNA fragments as they migrate through a polyacrylamide gel containing a linear gradient of DNA denaturants, usually urea and formamide. The exact composition of the gel, the gradient, and the amount of time that the gel runs must be optimized based on the melting behavior the sequences being analyzed [53, 54]. As the DNA is pulled through the gel by an electric current, there is an increase in the concentration of these denaturants. The stability of these fragments of DNA is based on the strength of the bonding between base pairs present in their sequence. Different fragments will therefore denature at a different concentration of denaturant, and a

different position within a lane. Guanine and cytosine (GC) base pairs exhibit more stable bonding than adenine and thymine (AT) base pairs, allowing GC-rich sequences to migrate further in a gel before becoming denatured [54]. It should be noted that other factors, such as the location of these base pairs in the fragment, also come into play in determining stability. This means that two sequences with the same ratio of base pairs will still have different stabilities, based on the location of these bases in the sequence. In order to prevent complete dissociation of DNA strands in the gel, DNA sequences for DGGE are amplified using primers that incorporate a GC clamp at one end of the product [53, 54]. The GC-rich clamp is a 30-50 string of GC base pairs that acts as an anchor, where the fragment will splay and stop migrating, forming a band on the gel. Because fragments from different species differ in their sequence, each band will, in theory, represent a different bacterial species [53, 54]. Banding patterns are visualized by staining gels with dyes such as ethidium bromide, or with SYBR green 1, which provides less background staining and may allow for better visualization of less pronounced bands [53]. DGGE is therefore a powerful tool in the comparison of many different samples at a single time. It is especially useful in studying the behavior of bacterial communities over a given time or as a result of different conditions, such as nutrient availability. DGGE is however limited in its ability to resolve very similar sequences, which often migrate so closely that their bands are superimposed. Vallaeys *et al.* showed that it is difficult or impossible to resolve 16S fragments with a very small amount of sequence variation [57]. Additionally, DGGE only gives a relative idea of the numbers of the bacteria present through the intensity of the bands present. Techniques

such as qPCR must be utilized in order to quantify bacteria of interest in an initial sample.

qPCR Is a method used to detect the relative or absolute abundance of specific sequence during amplification [58]. While PCR uses universal primers, qPCR uses primers specific to a sequences of interest in the genome. qPCR is one of the most widely used techniques for culture-independent quantification of bacteria in the feces and has been shown to be about 10-100 fold more sensitive than culture and fluorescence in situ hybridization [58]. qPCR is especially efficient when quantifying small amounts of DNA. qPCR follows the same scheme as PCR, but has the added feature of allowing DNA to be quantified in real time as it is being amplified. This is made possible by the use of dyes such as non-specific dyes that stain any double stranded DNA formed or sequence-specific DNA probes labeled with a fluorescent reporter that will permit detection only after hybridization [56]. Initially the quantity of sample will be too low to quantify, but there will reach a point at which a threshold value is reached. Quantification of DNA is based on the number of cycles required to reach this threshold intensity, denoted C_t. The greater the amount of initial product, the sooner this threshold value will be reached. If it is assumed that each sequence is present in the genome in only a single copy then the copy number will approximate the number of bacterial genomes present in the initial sample [56].

Together, these techniques can be used to develop a good picture of the microbiota, but they are still limited. Each one of these techniques introduces a unique potential bias. Rochelle *et al.* demonstrated that variations in the handling of marine sediment samples, prior to DNA extraction, lead to differences in the types and the

diversity of sequences observed via 16S rRNA sequence analysis [59]. The DNA extraction process itself can introduce bias. This occurs as a result of problems with effective disruption of all bacterial cells present in each sample and also with removal of inhibitors of the PCR reaction [54]. PCR can be a significant source of errors as well. There may be differential or preferential amplification of genes due to template strands reannealing to one another, rather than annealing to primer [59] Despite the limitations of the molecular techniques used, analysis of the 16S rDNA appears to be an effective means of studying the microbiota.

CHAPTER 2
PURPOSE

There are little data regarding the intakes of fiber in individuals with chronic kidney disease. These individuals may have reduced fiber intakes from whole grains, fruits, vegetables and legumes due poor appetite and dietary restrictions. Evidence suggests that increased fiber intake is associated with lowered blood urea nitrogen levels in individuals with compromised renal function, this may be associated with changes in the activity and composition of the microbiota. There have been no studies that use modern molecular tools to assess the changes in the microbiota as a result of fiber supplementation in individuals with chronic kidney disease. The overall objective of this study is to use these molecular tools to determine whether increasing intake of functional fiber in individuals with chronic kidney disease will lead to an increase in bacteria associated with health benefits, specifically lactic acid bacteria and bifidobacteria. We also wanted to determine what overall changes in diversity and composition would occur in these individuals as a result of increased functional fiber consumption.

CHAPTER 3
METHODS

Study Design

A six-week, single-blind, crossover study was carried out with 14 adults having a mean age of 64.8±14.7 years, all having chronic kidney disease. The study took part in two phases; a two-week control period, followed by a four-week treatment period. Participants were provided 4 servings/d of commercially-available food products containing low fiber during the control period, and containing added fiber during the treatment period. These foods were removed from their packaging and repackaged to prevent identification by study participants. Study foods included snack bars, cookies, and cereal containing added fiber and no added fiber. High-fiber and low-fiber foods were matched as closely as possible in taste and appearance. Participants provided two fecal samples throughout the study, during the second and sixth weeks of the study. Compliance was measured using daily diaries and confirmed by weighing leftover food returned upon completion of the study. Approval was obtained from the Institutional Review Board at the University of Florida, IRB-01. Informed consent was obtained from all the study participants.

Inclusion/ Exclusion Criteria after Obtaining Consent

Participants were included if they were 18 years of age and had chronic kidney disease, but were not on dialysis, and were willing to consume four different study foods each day for six weeks. Participants were excluded if they i) had been diagnosed with acute kidney injury, glomerulonephritis, or acute renal failure (ARF) over the past 3 months. ii) were scheduled to have a renal transplant or dialysis within 3 months of the study initiation, had a history of liver disease, dialysis, or had undergone renal

transplantation. ii) were on motility-stimulating drugs, steroids, immunosuppressant medication, or were unwilling to discontinue taking any prebiotics, fiber supplements, or laxatives iv) were pregnant, lactating, or had active gastrointestinal bleeding.

Pre-Baseline and Baseline

During the first study visit, participants were given a two-week supply of study food, and were taught to use the stool collection apparatus. Study food was packaged in plastic bags labeled with the day that food should be consumed. Participants were asked to return any leftover food in these plastic bags during their next study visit. Participants were also given a small Styrofoam cooler containing a stool collection kit and instructions for its use. Study personnel then gave a brief tutorial to each study participant, demonstrating how to assemble and use the kit. Participants were asked to call the study coordinator as soon as possible after providing a sample so that study personnel could retrieve it within four hours of defecation. Participants were informed that all samples should be kept on ice, but not frozen while waiting for pickup. During the first few days of the second week of the study, study coordinators made reminder calls to all participants asking that they provide a sample within the last three days of that week. This procedure was repeated for the treatment fecal samples. Participants were given another brief orientation during their penultimate study visit, reminding them how to use the stool collection apparatus. Participants were also called during the early part of week six, reminding them to provide a fecal sample at the end of that week. All samples were collected, homogenized, put into three separate tubes, and frozen at 70°C within 6 hours of defecation.

Treatment

Study participants were asked to consume two small cookies, a cereal bar, and two servings of cereal each day. During the intervention period study foods provided 23 grams per day of functional fiber, pea hull, inulin, and soluble corn fiber. During both the control and intervention weeks, food was packaged and labeled by date, to control serving size and prevent confusion. At each study visit, every two weeks starting at baseline, participants were provided with a two-week supply of packaged food. During the control period these foods included Kellogg's® Corn Pops®, Publix Chocolate chip cookies, and Kellogg's® Special K® bars. Each of these foods provided less than 1 gram of fiber per serving. Treatment foods included Kellogg's Corn Pops with fiber, Weight Watchers'® chocolate chip cookies, and Fiber One® bars. The fiber sources in each of the study foods can be found in tables 3-1 and 3-2 and the nutrient in each serving can be found in tables 3-3 and 3-4. Each day participants filled out surveys of compliance, listing which foods were consumed or omitted. Participants were also asked to return any food that was not consumed, as a check of self-reported compliance. Study personnel called all study participants weekly to maintain contact, even during weeks between study visits.

Daily and Weekly Measures

Participants were given daily diaries in which they answered brief questions about compliance and gastrointestinal symptoms. Participants were also asked to report their food intake for three days, four times throughout the study period. Daily diaries had perforated pages to allow participants to leave pages with study personnel during each visit.

Collection and Processing of Fecal Samples

Collection of two full defecations for microbial analysis occurred during the second and sixth weeks of the study. Samples were collected at home by study participants using a stool collection apparatus provided to them during their study visit. Samples were stored in a cooler of ice until processing. Study personnel retrieved and processed samples within six hours of defecation in order to ensure that handling of all samples was as standardized as possible. Prior to freezing, all samples were homogenized thoroughly. After processing, samples were frozen at -70°C until analysis.

Microbiota Analyses

Bacterial DNA was extracted from 200-300 mg fecal samples using a Quiagen stool DNA extraction kit. The protocol was modified to include a three-minute bead-beating step. PCR using universal primers was then used to amplify a 457-bp fragment from the V6 to V8 hypervariable region of the 16S ribosomal DNA. The PCR product was then analyzed by denaturing gradient gel electrophoresis, using an 8% (weight/volume) polyacrylamide gel with a denaturing gradient that increased from 40% at the top of the gel to 50% at the bottom (A 100% denaturing solution contains 40% (vol/vol) formamide and 7 M urea.). Samples were run at a constant voltage of 65 volts for 960 minutes at 60^{o}C. Gels were then stained with SYBR Gold and were scanned with Quantity One®, then analyzed with Diversity Database software.

Diversity of the bacterial populations, a measure of species richness and composition, was determined from DGGE analysis using the Shannon Wiener and Inverse Simpson diversity indices. Diversity indices increase when the number of

species in a population increases, reaching maximum values when all of the species within a population have the same number of individuals.

Bifidobacterium spp. genus, and *Lactobacillus spp*. genus were quantified using quantitative real-time polymerase chain reaction (qPCR). Specific primers for *Bifidobacterium spp.* genus and *Lactobacillus spp*. genus were used. qPCR analysis was performed in duplicate and 0.2μM primers. qPCR conditions used were: 10 min at 95°C followed by 40 cycles of 95°C for 30 s, annealing for 1 min and extension at 72°C for 30 s. Proportions of lactic acid and bifidobacteria were obtained by dividing the number positive for the primer by the total number of bacteria, obtained using the universal V3 primer set.

Lastly, DNA was amplified for sequencing using barcoded primers. PCR products were cleaned, pooled into equimolar amounts and submitted for sequencing. The resultant sequences were analyzed using the Ribosomal Database Project (RDP). Sequences were then grouped, or binned into operational taxonomic units (OTUs) by degrees of similarity. Using this binned sequence data, the structure and the diversity of the microbiota were analyzed using microbial ecology tools available in the Quantitative insights into Microbial Ecology (QIME) package.

Statistics

A paired t-test was used to calculate means and variation and for establishing two-sided significance levels ($p < 0.05$).The QIME package was used to calculate p-values for differences in UniFrac® distances.

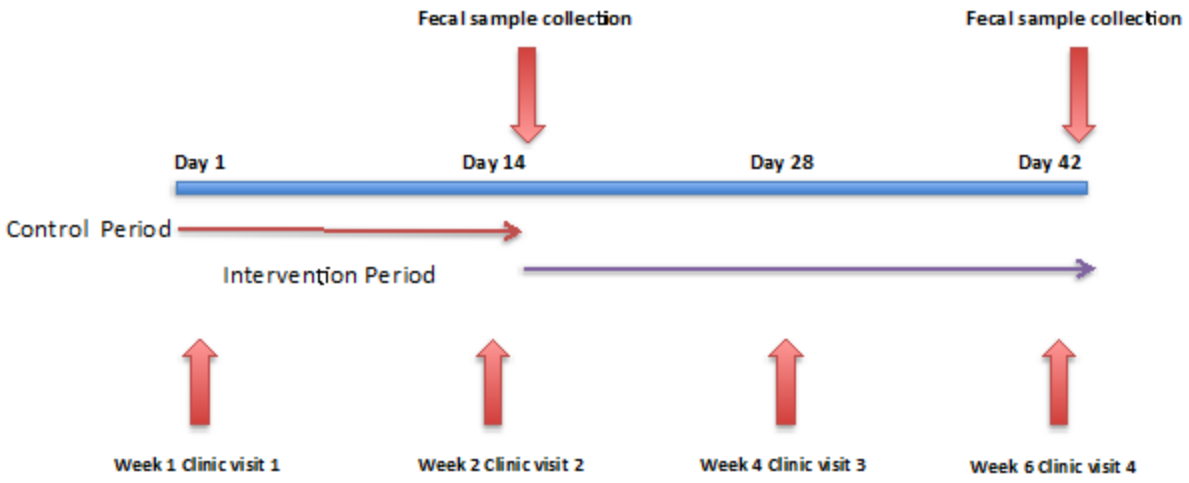


Figure 3-1. Study timeline.

Table 3-1. Content and source of fibers in foods provided during weeks 1 and 2

Food	Servings Provided	Fiber Content	Fiber source
Kellogg's® Corn Pops®	2	0g	N/A
Publix Chocolate Chip Cookies	2	0g	N/A
Kellogg's® Special K® Bars	1	<1g	N/A

Table 3-2. Content and source of fiber in foods Provided during weeks 3,4, and 5

Food	Servings Provided	Fiber Content	Fiber source
Kelloggs® Corn Pops® with Fiber	2	3g	Corn dextrin
Weight Watchers® Chocolate Chip Cookies	2	4g	Pea hull fiber
Fiber One® bars	1	9g	Chicory root

Table 3-3. Nutrient per serving for control foods

Food	Calories	Total fat	Protein	Carbohydrate
Kellogg's® Corn Pops®	110	0g	1g	26g
Publix Chocolate Chip Cookies	110	4g	<1g	8.5g
Kellogg's® Special K® Bars	90	1.5g	1g	17g

Table 3-4. Nutrient per serving for treatment foods

Food	Calories	Total fat	Protein	Carbohydrate
Kelloggs® Corn Pops® with Fiber	120	0g	1g	29g
Weight Watchers® Chocolate Chip Cookies	180	5g	2g	18g
Fiber One® bars	140	4g	2g	29g

CHAPTER 4
RESULTS

Subject Demographics and Characteristics

From an unknown population of individuals with chronic kidney disease being treated at Shands hospital in Gainesville, Florida, 270 were screened, 203 did not fit the inclusion criteria or declined contact. The 67 remaining individuals were contacted and 50 were excluded with secondary exclusion criteria or declined participation. Ultimately, 17 individuals were consented, and enrolled in the study, but only 14 provided the two fecal samples necessary for analysis. The other two were unable to produce samples within the necessary time frame and were therefore excluded. Of these 14 study participants, 9 were female and 6 were male; their mean age was 64.8±17.4. In the preliminary surveys, 11 participants reported white as their race, 7 of which non-Hispanic. The remaining 3 participants identified as black and non-Hispanic. Seven of the 14 participants who completed the study also had diabetes. Compliance was measured using self-reported information from daily diaries and also from the measure of leftover food returned by participants at each study visit. Compliance was 83% for the control period, and 78% for the treatment period. Self-reported data from 3-day food records filled out by participants throughout the study (data not included) indicated that mean fiber intake increased from 11.8±3.2 to 22.4±7.0 g/d ($p<0.001$) with no change in energy or protein intake.

Diversity and Structure of the Microbiota from DGGE

Comparisons of the baseline and treatment samples by qualitative DGGE profiling revealed that there were no significant changes in diversity between the two time points, as measured by the mean Shannon-Wiener diversity and inverse Simpson

Indices (Table 4-2). The average Shannon index for baseline samples, taken just prior to fiber supplementation, was 2.71 with a range of 2.48 to 2.94 while the average Shannon index at week six, after four weeks of consumption of fiber foods, was 2.64, with a range of 2.00 to 3.09. The p-value for the t-test between these two averages was 0.44, indicating no significant difference. Next, the average inverse Simpson Index was calculated for baseline and treatment samples. The average inverse Simpson index at baseline was 13.6 with a range of 11.9 to 18.7, while the value at treatment was 14.3 with a range of 9.9 to 27.7. This also showed no significance, with a t-test p-value of 0.611.

The dendrogram in Figure 4-2 is a graphical representation of the similarity coefficients between individuals. These values are obtained using software that measures the number and intensity of DGGE bands for each individual before and after treatment. Individuals with fewer nodes and shorter branch lengths between them are considered more closely related. Overall, the dendrogram does not show a strong grouping of participants based on treatment group. While some participants are more closely related to themselves before and after treatment than they are to participants of the same treatment group, others seem to differ significantly, making the results inconclusive.

Analysis of Bifidobacteria and Lactic Acid Bacteria

qPCR analysis at the two time points revealed that the mean number of genome equivalents at baseline was 63,100, while at treatment it was 127,682, Figure 4-5. The t-test p-value for this change did not reach significance ($p = 0.36$). The mean genome equivalents for the lactic acid bacteria decreased from 169,000 to 31,722 from baseline

to treatment (Figure 4-6), but also failed to reach significance with a t-test p-value of 0.39. These values were standardized against the total number of sequences to get the mean proportions of baseline genome equivalents. The Baseline mean proportions of genome equivalents for the bifidobacteria increased from 0.03 to 0.07 and also failed to reach significance ($p = 0.29$). Mean proportions of genome equivalents for lactic acid bacteria decreased from 0.035 to 0.013 ($p = 0.39$). These results indicate that there was no significant change in the lactic acid bacteria or bifidobacteria due to treatment.

Despite the lack of overall significance, 6 of our 14 study participants experienced a notable increase in the mean genome equivalents for Bifidobacteria due to fiber intervention, defined as a two or greater fold change. Of the remaining 8 study participants, 6 experienced a notable decrease in the Bifidobacteria genome equivalents and two experienced no change. Eight of the study participants experienced a notable increase in the genome equivalence of lactic acid bacteria, and 3 experienced a decrease. Three of the study participants experienced no change in lactic acid bacteria. Figure 4-3 and Figure 4-4 show the individual average genome equivalents for bifidobacteria and lactic acid, respectively.

Diversity and Structure from Sequence Analysis

The total numbers of OTUs present, as estimated using Chao1-based diversity plot curves, did not differ between control and treatment time points, shown in Fig 4-7. This can be seen by the overlap of the two curves, indicating that diversity was not significantly different between the two time points. Further analysis was done using the unique fraction metric (UniFrac) which computes differences between microbial communities based on phylogenetic differences between them, identified from 16S

sequences. This analysis also detected no change in diversity between the two time points. Based on the clustering and distribution of the control and treatment samples in principal component analysis. In Fig 4-6 it can be seen that populations do not appear to differentiate based on intervention.

Comparison of the distribution of OTUs between control and treatment samples revealed no trend in the change in the structure due to treatment. Figure 4-5 it indicates that there were however changes in the proportions of participants possessing specific OTUs.

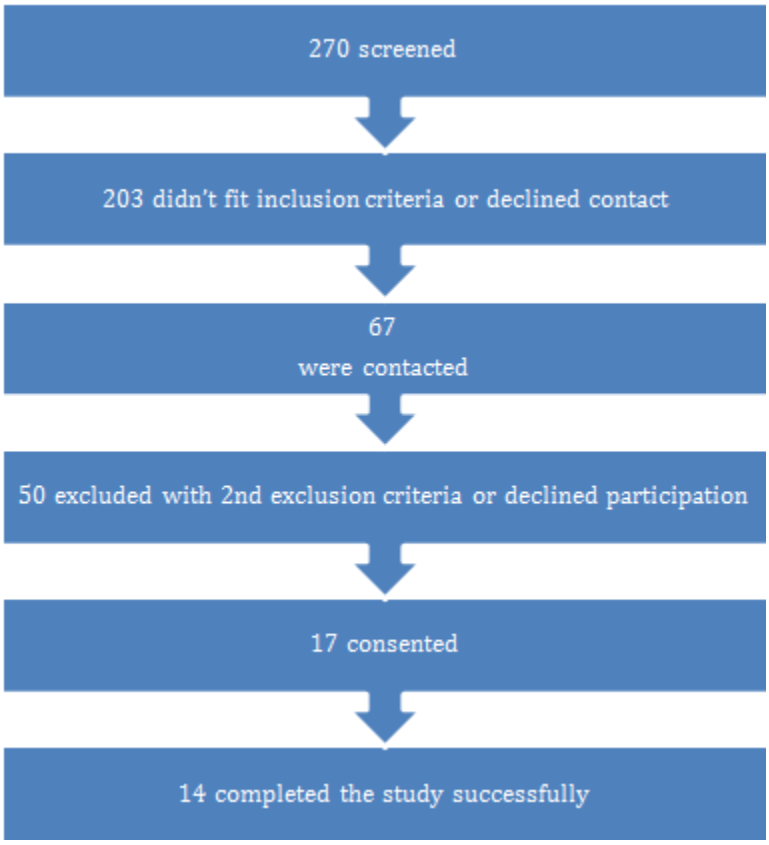


Figure 4-1. Flow chart of subject recruitment

Table 4-1. Mean fiber intake by fiber type (g/d)

Corn dextrin	Pea hull fiber	Fructooligosaccharide
3.8	6.2	7.0

Table 4-2. Average diversity of control and treatment samples measured by the Shannon index and inverse Simpson indices and their t-test p-values.

Statistical test	Control	Treatment	t-test p-value
Average Shannon index	2.71	2.64	0.44
Average inverse Simpson index	13.63	14.29	0.61

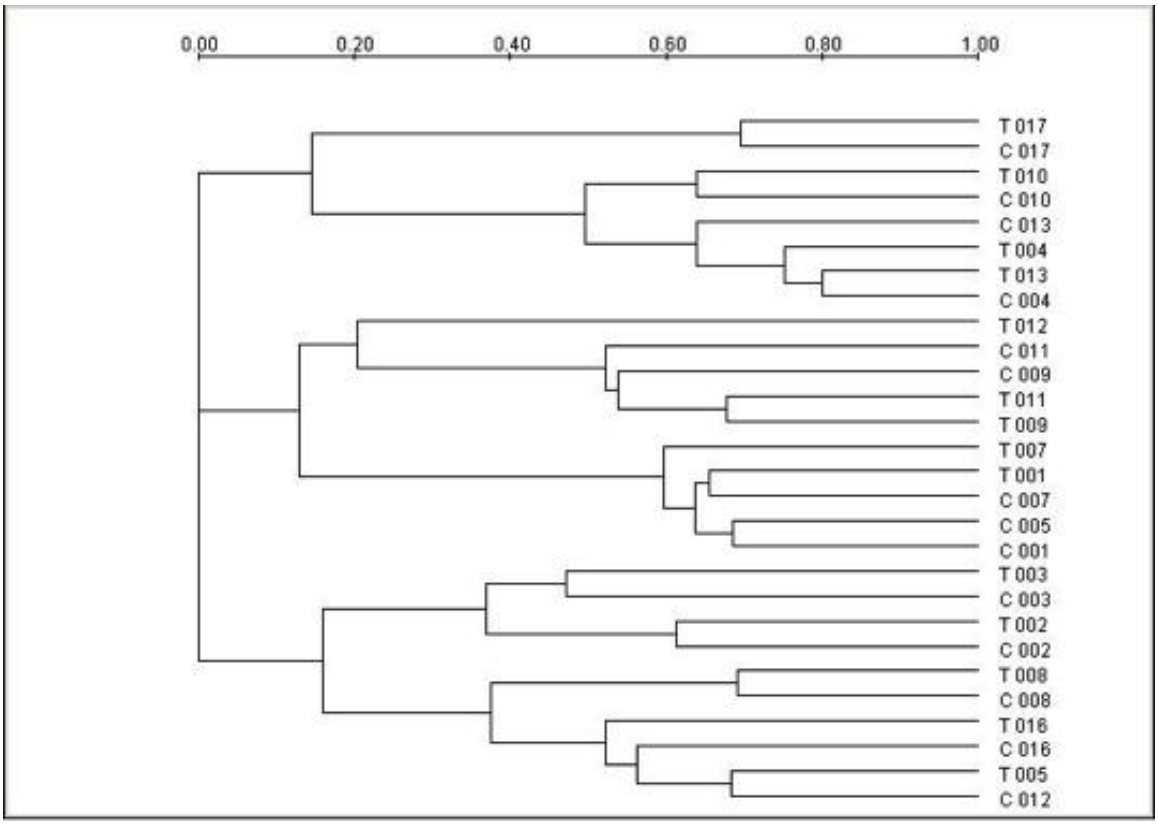


Figure 4-2. Dendrogram showing diversity based on DGGE analysis. C denotes control period and T denotes treatment, with the number corresponding to subject number. The similarity of samples is determined by the number of nodes between them and branch length, with fewer nodes signifying more similar diversity values.

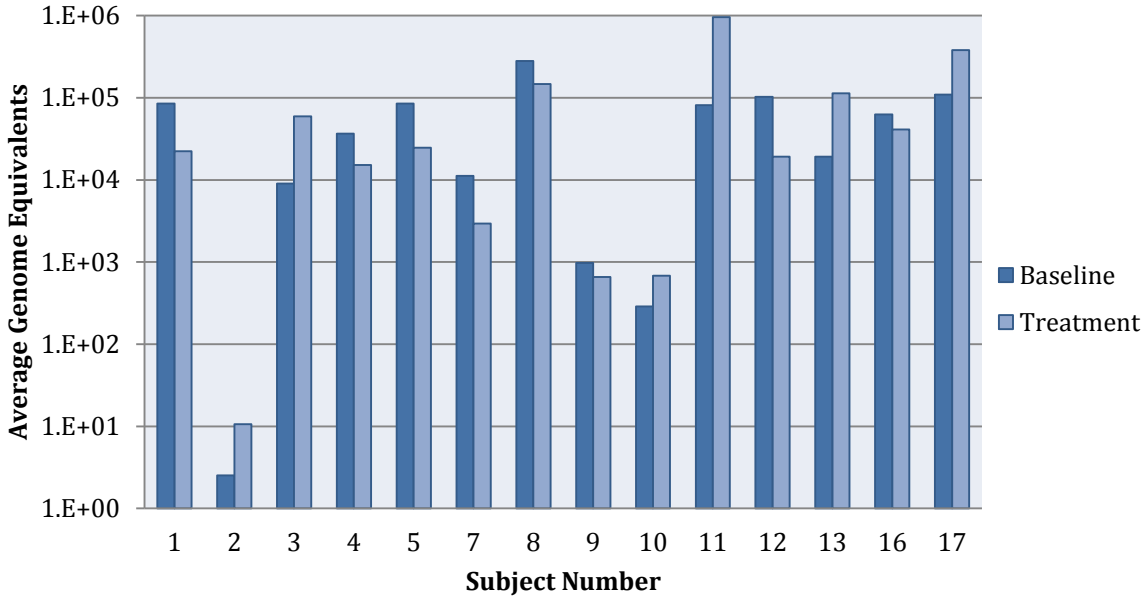


Figure 4-3. Mean Bifidobacteria genome equivalents in baseline and treatment samples

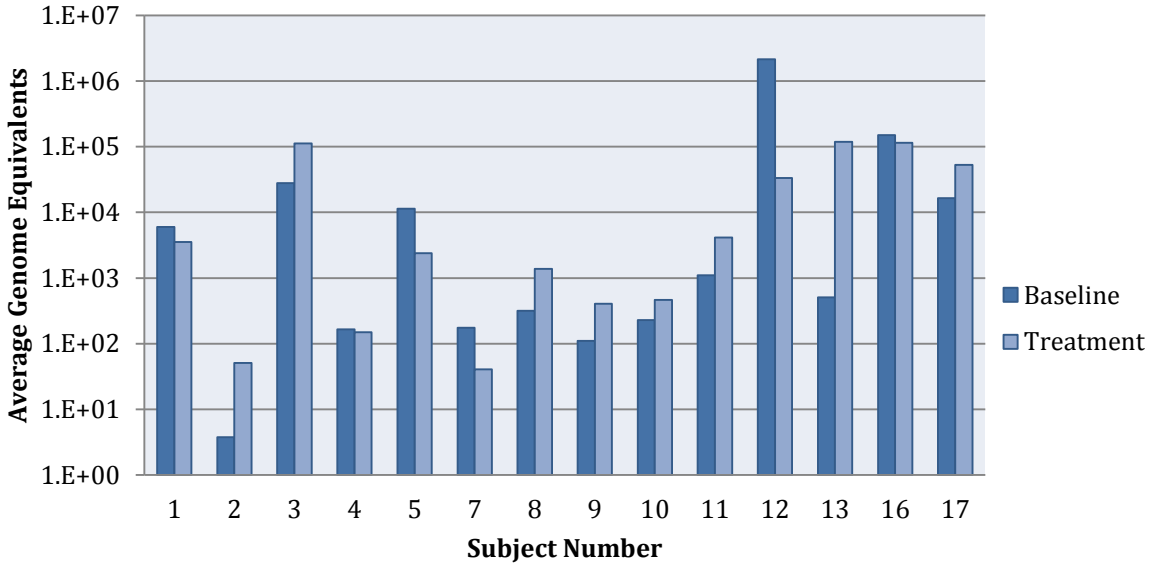


Figure 4-4. Mean lactic acid bacteria genome equivalents in baseline and treatment samples

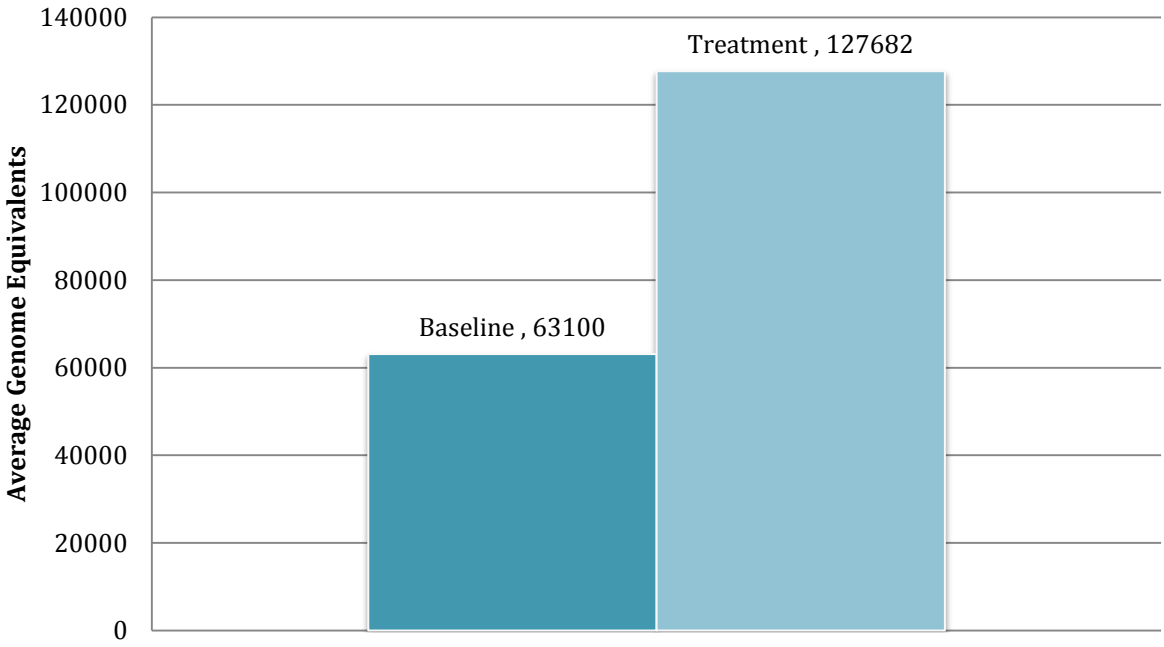


Figure 4-5. Average genome equivalents for bifidobacteria in baseline and treatment samples as measured by qPCR analysis.

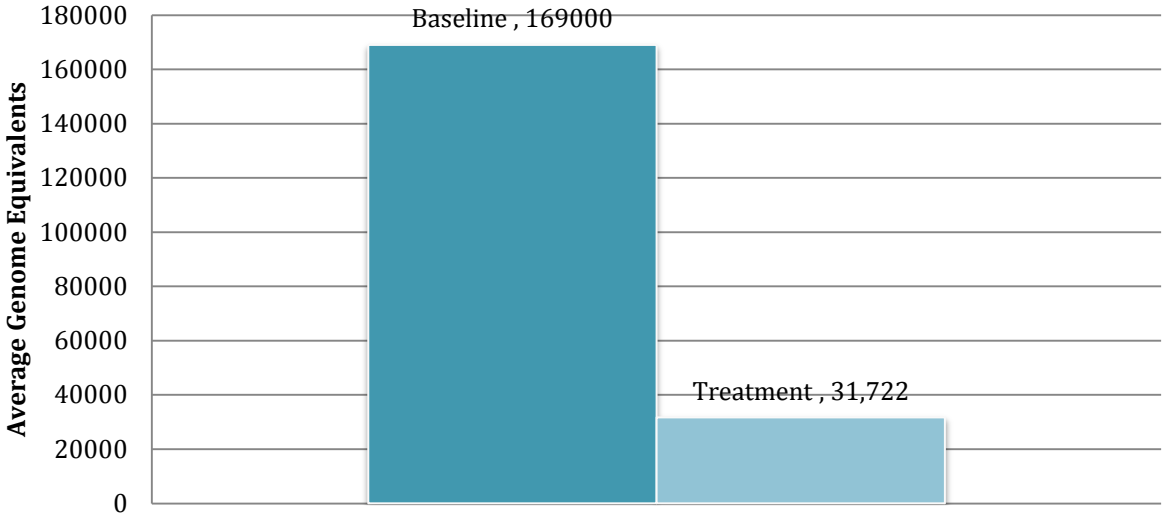
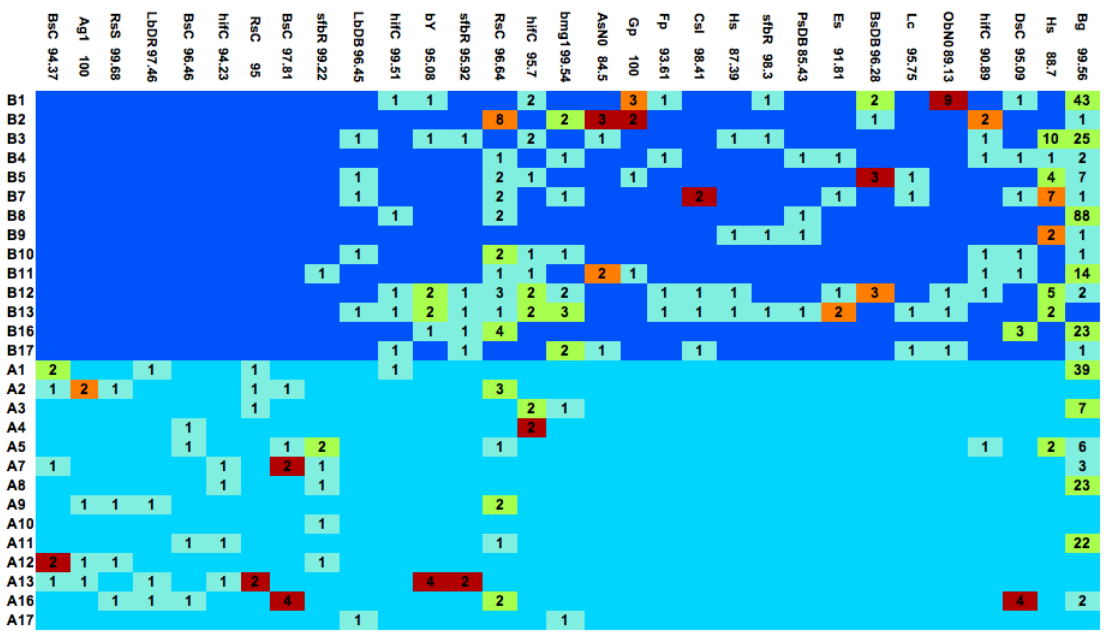


Figure 4-6. Average genome equivalents for lactic acid bacteria in baseline and treatment samples as measured by qPCR analysis.

A



B

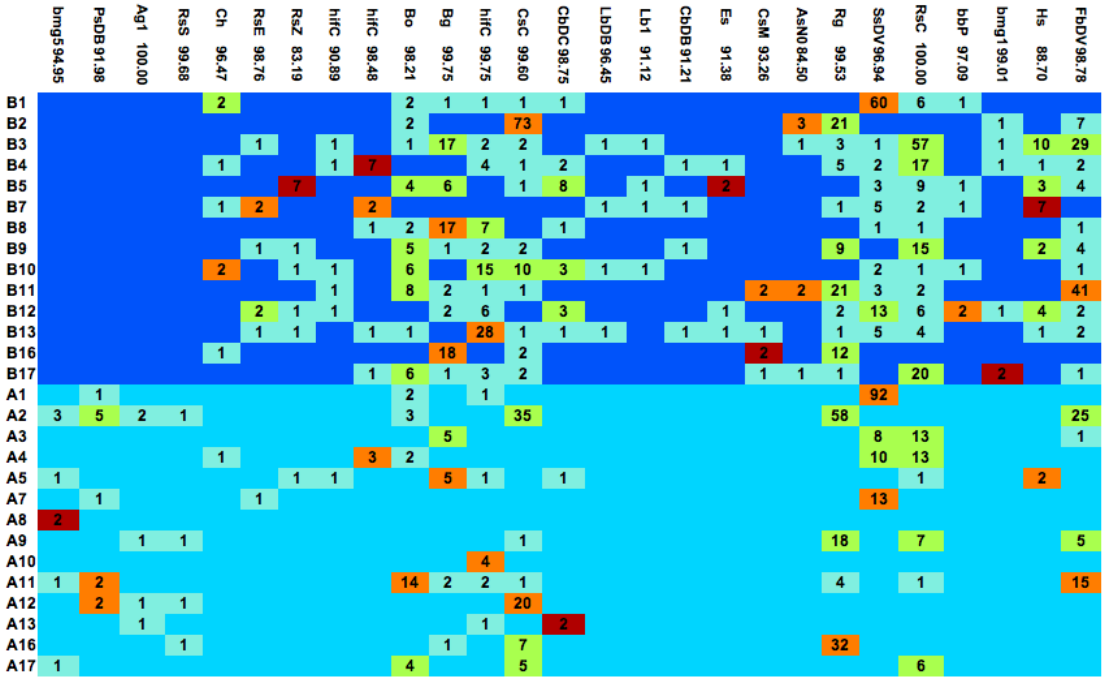


Figure 4-7. Differences in OTU abundance. Heat map of the top ranked OTUs for the 95% a) and 98% b). Rows denote subject number and columns denote OTUs, labeled with taxonomical abbreviations and percentage that the representative sequence matches to a database reference sequence. Colored squares denote the number of sequences within each bin, separated by subject and time point. B denotes before fiber and A denotes after.

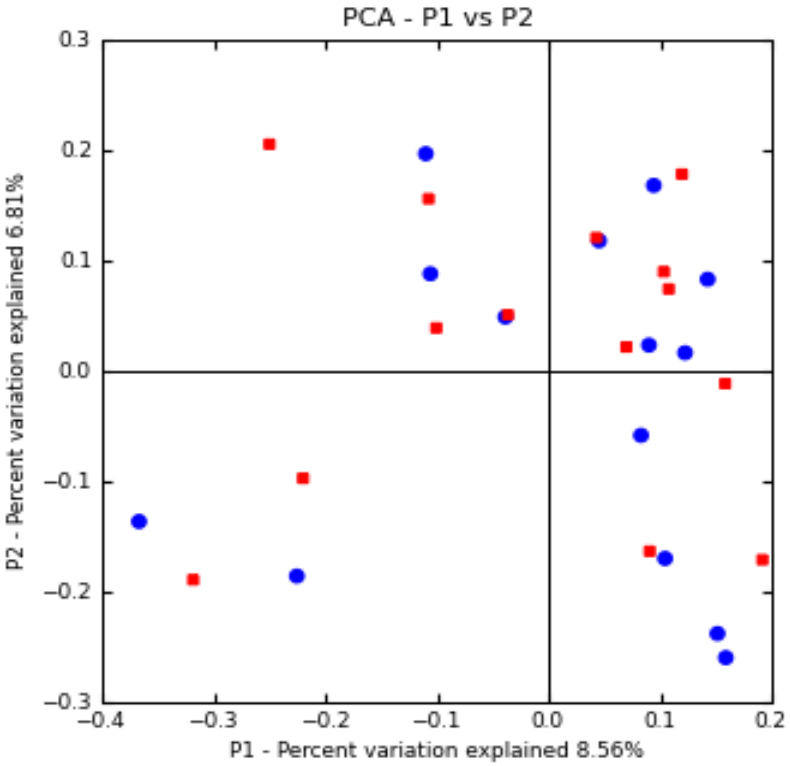


Figure 4-8. Unifrac diversity measures. Principal component analysis (PCA) of overall diversity based on Unifrac metric after fiber intervention. Blue circles represent baseline samples and red squares represent treatment samples.

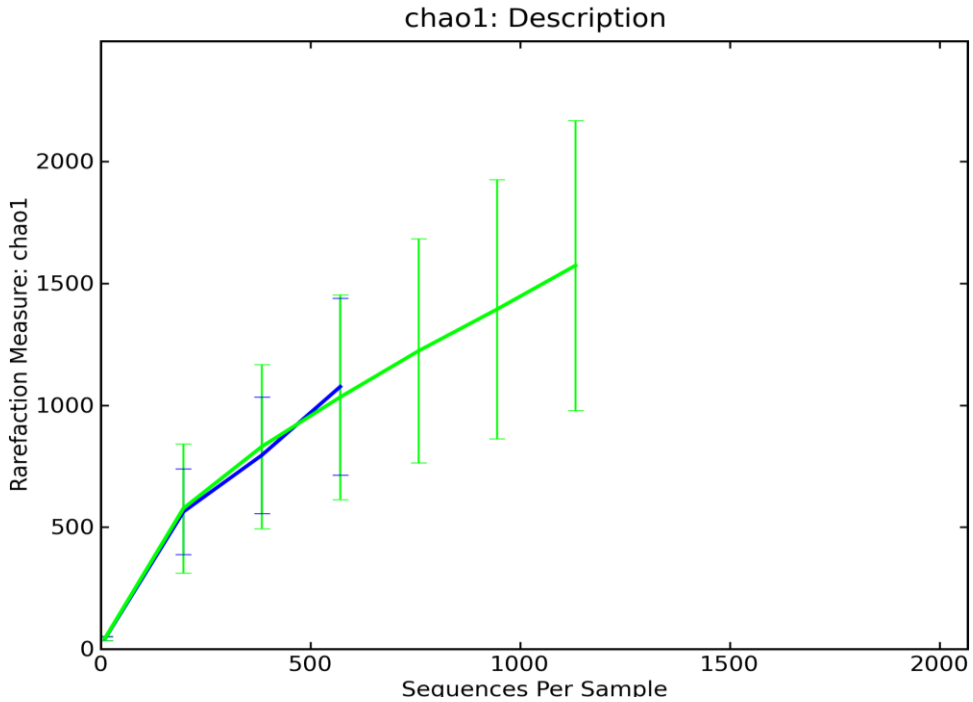


Figure 4-9. Chao diversity plot. Chao diversity was calculated from sequence distribution after 4 weeks of fiber intervention. Blue line represents baseline diversity and the green line indicates diversity for treatment samples.

CHAPTER 5
CONCLUSION AND DISCUSSION

In this study, individuals with CKD consumed an average of 16.5 grams/d of functional fiber in commercially-available food products for 4 weeks. Fecal samples were taken for microbiota analysis at baseline and at the end of the 4-week intervention period. As expected, average fiber consumption at baseline was lower than recommendations in this population. Based on self-reported data, total fiber Intake increased significantly in these individuals during the treatment period from 11.8 $\pm$ 3.2 to 22.4 $\pm$ 7g/d ($p<0.001$). This change was not associated with changes in the consumption of energy or protein.

Despite the significant increase in fiber intake in our study participants, the richness and distribution of the species in their microbiota did not change. This was measured both by DGGE and DNA sequence analysis. Increased functional fiber intake also had no significant impact on the overall levels of lactic acid bacteria or bifidobacteria in our study population, as measured by qPCR analysis. Despite these overall negative results, there were changes in the proportions of participants possessing specific OTUs as a result of fiber intake. These changes could not be characterized into intervention-specific changes and did not include bacteria that are well known for their beneficial or detrimental properties.

Study participants also experienced changes in bifidobacteria and lactic acid bacteria genome equivalents as a result of fiber intervention. While some participants experienced increases in mean genome equivalents of lactic acid bacteria and bifidobacteria others experienced decreases. There was no overall change seen across all study participants and changes across individuals did not appear to be related to the

significant decreases in BUN seen in the larger study by Salmean *et al.* Small sample size may have prevented accurate analysis of the correlation between changes in genome equivalents for these bacteria and changes in blood urea nitrogen levels.

When considering these findings, it is important to keep in mind that the microbiota is not well understood and that the relationship between changes in the microbiota and dietary intake is both complex and dynamic. Our results may also have been complicated by a number of issues with the study design. Issues with fiber sources, compliance, and the limitations of the molecular tools used may limit our analysis.

One of the major complicating factors in the analysis of these findings was the use of multiple fiber sources. Different fibers can selectively enhance the growth of particular bacteria in the microflora. Oligofructose derived from chicory root, present in the Fiber One® bars, selectively stimulates the growth and activity of bifidobacteria. Corn dextrin, present in the Kellogg's Corn Pops® would be expected to stimulate lactic acid bacteria growth. The insoluble pea hull fiber present in the Weight Watchers® Chocolate Chip Cookies is thought to be resistant to fermentation by the microbiota, but may decrease transit time, which may impact fermentation.

Examination of the compliance of each individual fiber provided during the treatment periods reveals that compliance was not uniform for all fiber sources. Average consumption of corn dextrin was only 3.8 g/d, likely insufficient to cause a significant increase in lactic acid bacteria. Alternatively, consumption of fructooligosaccharide and pea hull fiber was 7 and 6 g/d respectively. In a study by Bouhnik *et al* it was shown that the ideal oligofructose consumption for increased bifidobacteria counts, without side effects, was 10 grams per day [60]. The differences in compliance between the different

fiber sources may be attributed to volume of food that was provided to study participants for daily consumption.

Issues with appetite and changes in taste are common in the later stages of CKD. This may have made it difficult for our participants to fit the required servings of food into their diet. The length of the study, which was prolonged by the two-week control period, may have also caused fatigue as our study food stayed relatively consistent for the entire 6 weeks of the study. Additionally, restrictions on dairy product consumption for individuals with chronic kidney disease requires the use of milk alternatives that may be displeasing in taste, perhaps adding to poor cereal compliance. Rice milk was provided during this study, though all study participants did not use it. Half of the study participants had a diagnosis of diabetes, making it difficult to fit carbohydrate rich foods into their already limited diet.

Furthermore, the molecular techniques used in this study do not measure changes in microbial activity. This limits our view of the changes in the microbiota to presence or absence of bacterial sequences, exclusively. This is an important concept because metabolism in the colon is dependent on multiple bacterial species working with a number of saccharolytic enzymes. Bacteria can be classified as primary fermenters of carbohydrate or protein, but in reality their activity will vary based on available substrate. This makes categorizing bacteria as beneficial or detrimental largely speculative.

This study shows that the microbiota of individuals with chronic kidney can be changed with increased fiber consumption. However, the provision of mixed fiber sources, limited number of fecal samples collected, and issues with compliance make it difficult to characterize these changes. Future research should be done with this

population to determine how a single fiber source may impact the microbiota, and to determine whether a disease-specific CKD microbiota profile exists.

APPENDIX A
DOCUMENTS SUBMITTED TO THE IRB

UF Institutional Review Board
UNIVERSITY of FLORIDA

INFORMED CONSENT FORM
to Participate in Research, and
AUTHORIZATION
to Collect, Use, and Disclose Protected Health Information (PHI)

INTRODUCTION

Name of person seeking your consent:_____

Place of employment and position: _____
This is a research study of: the effects of fiber intake on gastrointestinal symptoms and quality of life in individuals with chronic kidney disease.
Could participating in this study offer any direct benefits to you? Yes, as described on page 5.

Could participating cause you any discomforts or are there any risks to you? Yes, as described on page 4.

Please read this form which describes the study in some detail. I or one of my co-workers will also describe this study to you and answer all of your questions. Your participation is entirely voluntary. If you choose to participate you can change your mind at any time and withdraw from the study. You will not be penalized in any way or lose any benefits to which you would otherwise be entitled if you choose not to participate in this study or to withdraw. If you have questions about your rights as a research subject, please call the University of Florida Institutional Review Board (IRB) office at (352) 846-1494. If you decide to take part in this study, please sign this form on page 10.

GENERAL INFORMATION ABOUT THIS STUDY

1. Name of Participant ("Study Subject")

_____

2. What is the Title of this research study?

An investigation into pulse fibre fermentation and nitrogen excretion in patients with chronic renal failure.

3. Who do you call if you have questions about this research study?

Dr. Wendy Dahl		Dr. Bobbi Langkamp-Henken	
work:	352-392-1991 ext. 224	work:	352-392-1991 ext. 205
cell:	352-226-1773	cell:	352-642-3669
home:	352-374-7798	home:	352-372-5434
email:	wdahl@ufl.edu	email:	henken@ufl.edu

4. Who is paying for this research study?

The sponsor of this study is Saskatchewan Pulse Growers, a Canadian commodity organization that promotes the sale of Saskatchewan (Canada) grown beans, peas and lentils.

5. Why is this research study being done?

The purpose of this research study is to determine if consuming fiber-fortified baked goods and cereal will improve the quality of life, gastrointestinal symptoms, appetite, blood values and other outcomes in individuals with chronic kidney disease.
You are being asked to be in this research study because you are a chronic kidney disease patient in stage 4 or 5 of the disease's progression.

WHAT CAN YOU EXPECT IF YOU PARTICIPATE IN THIS STUDY?

6. What will be done as part of your normal clinical care (even if you did not participate in this research study)?

This study is not related to your normal clinical care. Your nephrologist will not be informed that you are taking part in this study.

7. What will be done only because you are in this research study?

If you decide to take part in this study, you will be randomly assigned (much like the flip of a coin) to receive either baked goods and cereal fortified with fiber (experimental foods) or baked goods and cereal without fiber fortification (control foods). All participants will consume the unfortified foods for a two week baseline period. The control is a food that looks similar and is given in the same way as an experimental food but contains no fiber fortification. A control is used in nutrition research studies to show what effect a treatment has compared with taking nothing at all. If you are assigned to receive the control, you will not receive the benefits of the fiber-fortified foods, but will still receive similar nutrients and calories found in regular baked goods and cereal. Both groups are exposed to the same risks, which are described below under "What are the possible discomforts and risks?" You will not be told whether you are receiving control (regular baked goods and cereal) or the fiber-fortified foods, but that information is available if it is needed. Also, you will have a 50% chance of receiving the control foods and a 50% chance of receiving the fiber-fortified foods. In the remainder of the description of what will be done, both the control and the fiber-fortified foods will be called "study foods"

1. At the beginning of the study, you will have a study visit to the Food Science and Human Nutrition Clinical lab at the Food Science Human Nutrition building at the University of Florida. You will be asked to return for a study visit in weeks 2, 4 and the final week, week 6.

During each study visit:

2. A licensed phlebotomist will obtain 2 tsp. venous blood from you for assessment of BUN (blood urea nitrogen – a measurement of nitrogen wastes in your blood), creatinine (a measurement of kidney function), fasting blood glucose (blood sugar), and a cholesterol profile. This component of the study will take approximately 15 minutes to complete. You are required to fast a minimum of 6 hours prior to the blood draw and it is preferable if you fast overnight. The blood draw will take place in the blood draw room of the Food Science and Human Nutrition Clinical Laboratory
3. You will be asked to complete three questionnaires. A Simplified Nutritional Appetite Questionnaire (SNAQ), GSRS (Gastrointestinal Symptom Questionnaire and a quality of life questionnaire "Your Health– *and* – Well-Being Kidney Disease and Quality of Life (KDQOL™-36).
4. Body weight and height will be assessed on the first day of the study and at your final study visit. These procedures will take less than 10 minutes.
5. You will be given a week's supply of study foods at a time and asked to consume 3-5 servings of these foods each day for 6 weeks.

At home:

6. You will be given a study diary and asked to answer questions about medication, intake of the study foods and gastrointestinal function each day. You will also receive pre-dated bags to safe any left over study foods.
7. You will be asked to complete a 3-day food record in week 1 of the study and during week 6 of the study.
8. A study coordinator will visit you at your home during week 2, 3 and 5 of the study and will deliver your week's food as well as have you complete the GSRS questionnaire. Study coordinator will also pick up any left over study foods.
9. During week 1 and week 6, you will be asked to collect two stool samples each week. You will be provided with a collection container specially designed for stool collections. When collection is made, you will be required to call the study cell phone and a laboratory technician will pick up your sample from your home.

If you have any questions now or at any time during the study, please contact <insert name of PI> in question 3 of this form.

8. How long will you be in this research study?

You will be in this study for six weeks. The process of consent, the first blood sample and height and weight measurements and questionnaires are expected to take up to 1½ hours. Follow- up study visits are also expected to take up to 1 hour. Consuming the study foods are estimated to take 30 minutes per day, but are expected to replace usual foods you consume. Stool collections are expected to take 15 minutes per sample. Study diary completion will take less than 5 minutes per day. Home visits are expected to take 20 minutes per session. Food records are expected to take 1 hr per

day. Total study time, not including travel to and from study visits is expected to be less than 20 hrs not including time taken to consume study foods.

9. How many people are expected to take part in this research study?
Twenty people will be participating in this research study.

WHAT ARE THE RISKS AND BENEFITS OF THIS STUDY AND WHAT ARE YOUR OPTIONS?

10. What are the possible discomforts and risks from taking part in this research study?

- The risks of drawing blood from a vein include discomfort at the site of puncture; possible bruising and swelling around the puncture site; rarely an infection; and, uncommonly, faintness from the procedure.
- An overnight fast is required and may cause physical discomfort.
- Some people may feel uncomfortable when body weight is measured.
- Certain questions on the questionnaires may be personal and thus, may be upsetting. You may choose not to answer any of the questions.
- Consuming fiber-fortified foods may result in bloating, gas (flatulence) and abdominal discomfort.

Other possible risks to you may include: This study may include risks that are unknown at this time.

Participation in more than one research study or project may further increase the risks to you. If you are already enrolled in another research study, please inform <insert name of PI> (listed in question 3 of this consent form) or the person reviewing this consent with you before enrolling in this or any other research study or project.

Throughout the study, the researchers will notify you of new information that may become available and might affect your decision to remain in the study.

If you wish to discuss the information above or any discomforts you may experience, please ask questions now or call the PI or contact person listed on the front page of this form.

11a. What are the potential benefits to you for taking part in this research study?
You may or may not benefit from participating in this research study. If you have a low fiber intake you may benefit from increased intake of fiber if you are assigned to the fiber-fortified group. The foods provided to you in this study contain energy and other nutrients that may be important to health.

11b. How could others possibly benefit from this study?
Research into the efficacy of fiber-fortified food sources on the quality of life and wellness of individuals with chronic kidney disease may lead to revised diet instruction for this population.

11c. How could the researchers benefit from this study?

In general, presenting research results helps the career of a scientist. Therefore, <insert name of PI> may benefit if the results of this study are presented at scientific meetings or in scientific journals.

The researchers will receive no financial compensation for the completion of this study. The study sponsor will reimburse the researchers for study operating costs only.

12. What other choices do you have if you do not want to be in this study?

The option to participating is to do nothing – do not sign this consent form.

You have been invited to participate in this research project because you have chronic kidney disease in Stage 4 or 5 (pre-dialysis)**.** Your participation in this study is voluntary and any decision to take part or not to participate, will in no way affect your medical care.

13a. Can you withdraw from this study?

You are free to withdraw your consent and to stop participating in this study at any time. If you do withdraw your consent, you will not be penalized in any way and you will not lose any benefits to which you are entitled.

If you decide to withdraw your consent to participate in this study for any reason, please contact <insert name of PI or study coordinator> at 352-<phone number> or Dr. Langkamp-Henken at 352-392-1991 ext. 205. They will tell you how to stop your participation safely.

If you have any questions regarding your rights as a research subject, please call the Institutional Review Board (IRB) office at (352) 846-1494.

13b. If you withdraw, can information about you still be used and/or collected?

If you withdraw you consent, your information will not be used.

13c. Can the Principal Investigator withdraw you from this study?

You may be withdrawn from the study without your consent for the following reasons: You did not follow the instructions given, e.g. did not consume the study foods, answer the questionnaires or did not provide the blood samples.

WHAT ARE THE FINANCIAL ISSUES IF YOU PARTICIPATE?

14. If you choose to take part in this research study, will it cost you anything?

The sponsor will only pay for medical services that you receive as part of your participation in this study as described in question 7 above. All other medical services that you would have received if you were not in this study will be billed to you or your insurance company. You will be responsible for paying any deductible, co-insurance, co-payments for those services and for any non-covered or out –of –network services. Some insurance companies may not cover costs associated with studies. Please contact your insurance company for additional information.

15. Will you be paid for taking part in this study?

Yes, you will be paid $25 per week for a total of $125 for participating in the study.

If you are paid for taking part in this study, your name and social security number will be reported to the appropriate University employees for purposes of making and recording the payment. You are responsible for paying income taxes on any payments provided by the study. If the payments total $600 or more, the University must report the amount you received to the Internal Revenue Service (IRS).

16. What if you are injured because of the study?

If you are injured as a direct result of your participation in this study, the Sponsor will pay for all reasonable and necessary medical expenses required to treat your injury, as long as:

1. The injury occurs during the study and results directly from the Study-related procedures which you would not have received as part of your routine medical care;
2. The injury is not listed in question 10 above;
3. The injury is not the result of the natural course of your disease or some other underlying condition and;
4. Your insurance company denies payment for the medical services.

The Sponsor and the Principle Investigator will determine whether your injury is related to your participation in this study.

The Principle Investigator and others involved in this study may be University of Florida employees. As employees of the university, they are protected under state law, which limits financial recovery for negligence.

Please contact the Principal Investigator listed in question 3 of this form if you experience an injury or have questions about any discomforts that you experience while participating in this study.

17. How will your health information be collected, used and shared?

If you agree to participate in this study, the Principal Investigator will create, collect, and use private information about you and your health. This information is called protected health information or PHI. In order to do this, the Principal Investigator needs your authorization. The following section describes what PHI will be collected, used and shared, how it will be collected, used, and shared, who will collect, use or share it, who will have access to it, how it will be secured, and what your rights are to revoke this authorization.

Your protected health information may be collected, used, and shared with others to determine if you can participate in the study, and then as part of your participation in the study. This information can be gathered from you or your past, current or future health records, from procedures such as physical examinations, x-rays, blood or urine tests or from other procedures or tests. This information will be created by receiving study treatments or participating in study procedures, or from your study visits and telephone calls. More specifically, the following information may be collected, used, and shared with others:

- Your social security number for compensation purposes

- Body weight, height, age, sex, race and ethnicity
- Blood urea nitrogen, creatinine, fasting blood glucose, cholesterol profile
- Dietary intake
- Gastrointestinal function and symptoms
- Stool microbiota data
- Your responses to the three questionnaires “A Simplified Nutritional Appetite Questionnaire (SNAQ)”, “GSRS (Gastrointestinal Symptom Questionnaire)” “Your Health– *and* –Well-Being Kidney Disease and Quality of Life (KDQOL™-36).

This information will be stored in locked filing cabinets or on computer servers with secure passwords, or encrypted electronic storage devices.

Some of the information collected could be included in a "limited data set" to be used for other research purposes. If so, the limited data set will only include information that does not directly identify you. For example, the limited data set cannot include your name, address, telephone number, social security number, photographs, or other codes that link you to the information in the limited data set. If limited data sets are created and used, agreements between the parties creating and receiving the limited data set are required in order to protect your identity and confidentiality and privacy.

18. For what study-related purposes will your protected health information be collected, used, and shared with others?

Your PHI may be collected, used, and shared with others to make sure you can participate in the research, through your participation in the research, and to evaluate the results of the research study. More specifically, your PHI may be collected, used, and shared with others for the following study-related purpose(s):

- to examine the effects of consuming fiber fortification on quality of life and wellness of individuals with chronic kidney disease.

Once this information is collected, it becomes part of the research record for this study.

19. Who will be allowed to collect, use, and share your protected health information?

Only certain people have the legal right to collect, use and share your research records, and they will protect the privacy and security of these records to the extent the law allows. These people include the:

- the study Principal Investigators, <insert name of PI> and research staff and graduate students associated with this project.
- the University of Florida Institutional Review Board (IRB; an IRB is a group of people who are responsible for looking after the rights and welfare of people taking part in research).
- Other professionals at the University of Florida who provide study-related procedures.

20. Once collected or used, who may your protected health information be shared with?

Your PHI may be shared with:

- the study sponsor *Saskatchewan Pulse Growers*

- United States and foreign governmental agencies who are responsible for overseeing research, such as the Food and Drug Administration, the Department of Health and Human Services, and the Office of Human Research Protections
- Government agencies who are responsible for overseeing public health concerns such as the Centers for Disease Control and federal, state and local health departments

Otherwise, your research records will not be released without your permission unless required by law or a court order. It is possible that once this information is shared with authorized persons, it could be shared by the persons or agencies who receive it and it would no longer be protected by the federal medical privacy law.

21. If you agree to take part in this research study, how long will your protected health information be used and shared with others?

Your PHI will be used and shared with others for one year following the completion of the study.

You are not required to sign this consent and authorization or allow researchers to collect, use and share your PHI. Your refusal to sign will not affect your treatment, payment, enrollment, or eligibility for any benefits outside this research study. However, you cannot participate in this research unless you allow the collection, use and sharing of your protected health information by signing this consent and authorization.
You have the right to review and copy your protected health information. However, we can make this available only after the study is finished.
You can revoke your authorization at any time before, during, or after your participation in this study. If you revoke it, no new information will be collected about you. However, information that was already collected may still be used and shared with others if the researchers have relied on it to complete the research. You can revoke your authorization by giving a written request with your signature on it to the Principal Investigator.

SIGNATURES

As an investigator or the investigator's representative, I have explained to the participant the purpose, the procedures, the possible benefits, and the risks of this research study; the alternative to being in the study; and how the participant's protected health information will be collected, used, and shared with others:

Signature of Person Obtaining Consent and Authorization	Date

You have been informed about this study's purpose, procedures, possible benefits, and risks; the alternatives to being in the study; and how your protected health information will be collected, used and shared with others. You have received a copy of this Form. You have been given the opportunity to ask questions before you sign, and you have been told that you can ask questions at any time.

You voluntarily agree to participate in this study. You hereby authorize the collection, use and sharing of your protected health information as described in sections 17-21 above. By signing this form, you are not waiving any of your legal rights.

Signature of Person Consenting and Authorizing	Date

Appendix A

Daily Diary Date:_____ Study #_____

1. How many bowel movements did you have today?

❑ 0
❑ 1
❑ 2
❑ 3
❑ 4
❑ 5
❑ >6

2. Did you experience diarrhea today?

❑ yes
❑ no

3. Did you take laxative today?

❑ yes
❑ no

4. Are you currently taking antibiotics?

❑ yes
❑ no

5. Did your medication change?

❑ yes
❑ no

6. How many servings of study foods did you consume today?

❑ 0
❑ 1
❑ 2
❑ 3
❑ 4
❑ 5
❑ >6

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7. Did you consume a fiber supplement today? If so, what did you take?

Telephone Script

Hello, my name is_____ , with the fiber study.
(Potential participant indicates they are calling about the study)
Great. I would be happy to give you more information about the study. The purpose of this study is to determine whether the providing adequate fiber to patients with CKD will result in improved gastrointestinal function and quality of life.
If you qualify and decide to participate, you will be randomly assigned to a treatment or control group, but you will not be told which group you are in until completion of the study. Both groups will be given cookies, cereal bars and breakfast cereal and to consume daily for a period of 6 weeks (42 days), with the treatment group receiving high fiber food. During the course of the study you would be asked to come in our clinical lab on four separate occasions to have your blood drawn and fill out questionnaires. These appointments should take no more than an hour. The questionnaires will ask questions regarding your quality of life, appetite, and gastrointestinal symptoms. Participants will also be asked to provide three stool samples during the study.
Foods provided to participants provide nutrients and energy to all participants. Individuals selected for the fiber-fortification group may experience improved gastrointestinal function and quality of life due to the fiber. Does this sound like something you would be interested in doing?
(Responds Yes)
Great, now I will read the inclusion/exclusion criteria for the study to make sure you qualify. Please wait until I finish reading through this list, then you can let me know if you are still interested.
(Read inclusion/exclusion criteria without pausing)
Does this still sound like something you would like to take part in?
(Responds Yes)
Great, then we can schedule an initial appointment for you to receive more detailed information on the study and review a consent form. Is there a date and time that works best for you?
(Schedule appointment and obtain best way to contact patient)

Inclusion Criteria

Participants Must:

- Be 18 years of age or older
- Have GFR of ≤ 29 mL/min/1.73 m^2 (stage 4 and 5 but who are not on dialysis)

Exclusion Criteria

- Have you been diagnosed with acute kidney injury (AKI)
- Have you been diagnosed with glumerulonephritis (GN)?
- Are you on immunosuppressant/steroid medications?
- Are you taking a probiotic supplement and refuse to discontinue it?
- Are you scheduled for dialysis within 3 months of study initiation?
- Do you have a history of liver disease?
- Have you been on dialysis?
- Have you undergone renal transplantation?
- Are you breastfeeding?
- Do you have active gastrointestinal bleeding?
- Have a change in medications over the past 4 weeks?

If you have answered any of the above questions with “yes”, you are not eligible to take part in the study.

Are you still interested in taking part in the study?

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BIOGRAPHICAL SKETCH

Justin Forde was born and raised in Miami, FL where his family currently resides. he received his Bachelor of Science degree at the University of Florida in Gainesville, FL in 2009. He graduated with his Master of Science degree at the University of Florida in Gainesville, FL in the spring of 2012.