

## **The phosphate binder ferric citrate alters the gut microbiome in rats with chronic kidney disease**

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Running Title: Ferric citrate and gut microbiome in CKD rats

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Number of text pages: 14

Abstract word count: 245

Word count: 3,624

Introduction word count: 809

Discussion word count: 1,029

Tables: 1

Figures: 5

Supplemental Table: 1

Supplemental Figures: 2

Abbreviations:

BUN	blood urea nitrogen
CKD	chronic kidney disease
DNA	deoxyribonucleic acid
ESKD	end-stage kidney disease
FC	ferric citrate
NL	normal
OTUs	Operational Taxonomic Units
PERMANOVA	Permutational Multivariate Analysis of Variance
rRNA	ribosomal ribonucleic acid

## Abstract

In chronic kidney disease (CKD), the gut microbiome is altered and bacterial-derived uremic toxins promote systemic inflammation and cardiovascular disease. Ferric citrate complex is a dietary phosphate binder prescribed in end-stage kidney disease patients to treat hyperphosphatemia and secondary hyperparathyroidism. Iron is an essential nutrient in both microbes and mammals. The present study was undertaken to test the hypothesis that the large iron load administered with ferric citrate in CKD may significantly change the gut microbiome. Male Sprague Dawley rats underwent 5/6 nephrectomy to induce CKD. Normal control and CKD rats were randomized to regular chow or a 4% ferric citrate diet for 6 weeks. Fecal and cecal microbial DNA was analyzed via 16S rRNA gene sequencing on the Illumina MiSeq. CKD rats had lower abundances of Firmicutes and *Lactobacillus* compared to normal rats and had lower overall gut microbial diversity. CKD rats treated with ferric citrate had improved hemoglobin and creatinine clearance, and amelioration of hyperphosphatemia and hypertension. Ferric citrate treatment increased bacterial diversity in CKD rats almost to levels observed in control rats. The tryptophanase-possessing families Verrucomicrobia, Clostridiaceae and Enterobacteriaceae were increased by ferric citrate treatment. The uremic toxins indoxyl sulfate and *p*-cresyl sulfate were not increased with ferric citrate treatment. Verrucomicrobia was largely represented by *Akkermansia muciniphila* which has important roles in mucin degradation and gut barrier integrity. In summary, ferric citrate therapy in CKD rats was associated with significant changes in the gut microbiome and beneficial kidney and blood pressure parameters.

## Introduction

Systemic inflammation is invariably present in humans and animals with chronic kidney disease (CKD) and is marked by activation of circulating leukocytes and elevation of plasma pro-inflammatory cytokines and chemokines (Yoon et al., 2007; Kato et al., 2008; Heine et al., 2012). The CKD-associated systemic inflammation and oxidative stress play a central role in the pathogenesis of numerous complications of CKD including accelerated cardiovascular disease, frailty, and anemia, among others (Himmelfarb et al., 2002; Vaziri, 2004; Cachofeiro et al., 2008). There are profound changes in the structure and function of the gut microbiome and disruption of the gut epithelial barrier in CKD (Vaziri et al., 2012; Vaziri et al., 2013a; Vaziri et al., 2013b). Increased permeability of the intestinal epithelium in CKD is evidenced by the appearance of orally-administered high molecular weight polyethylene glycols in the urine (Magnusson et al., 1990; Magnusson et al., 1991). Several studies have demonstrated the role of gut microbial-derived uremic toxins such as indoxyl sulfate, *p*-cresyl sulfate and trimethylamine-N-oxide in the pathogenesis of CKD-associated systemic inflammation (Liabeuf et al., 2010; Aronov et al., 2011; Tang et al., 2015; Stubbs et al., 2016). Endotoxin, derived from the cell wall of Gram-negative bacteria, is measurable in the blood of CKD patients and increases with severity of CKD stage, being most elevated in chronic dialysis patients (Szeto et al., 2008; McIntyre et al., 2011).

Several factors contribute to the gut microbial dysbiosis in patients with advanced CKD. I- Accumulation of urea in the body fluids and its diffusion in the gastrointestinal tract leads to the expansion of urease-possessing bacteria (Wong et al., 2014). Hydrolysis of urea by these microbial species results in formation of ammonia and caustic ammonium hydroxide [CO(NH<sub>2</sub>)<sub>2</sub>

+ H<sub>2</sub>O → CO<sub>2</sub> + 2NH<sub>3</sub>; NH<sub>3</sub> + H<sub>2</sub>O → NH<sub>4</sub>OH], which degrade the epithelial tight junction (Vaziri et al., 2013c) thereby facilitating translocation of endotoxin and microbial fragments into the systemic circulation (Wang et al., 2012; Lau et al., 2015; Vaziri et al., 2016). **II-** Dietary restrictions to limit hyperkalemia and hyperphosphatemia in CKD correlates with a diet low in potassium-rich (fruits and vegetables) and phosphate-rich (cheese and yogurts) products. These dietary restrictions result in the unintended reduction of indigestible complex carbohydrates, a nutrient source for gut microbial flora, from which they generate short chain fatty acids. Short chain fatty acids in turn are major nutrients for colonocytes and are essential for the integrity of the colonic epithelium. In addition, the reduction of short chain fatty acid production and formation of ammonium hydroxide leads to a rise in the pH of the colonic milieu which further impacts the gut microbial community (Sirich et al., 2014; Kieffer et al., 2016). Further, the limited intake of cheese and yogurt reduces exposure to useful symbiotic bacteria. **III-** Pharmaceutical interventions including commonly prescribed phosphate or potassium binders significantly alter the biochemical milieu of the gut and can potentially impact the gut microbiome. However, to our knowledge the effect of these compounds on the gut microbiome in CKD has not been previously investigated.

Ferric citrate is a calcium-free iron compound used as a dietary phosphorus binder to manage hyperphosphatemia in end-stage kidney disease (ESKD) patients (Yokoyama et al., 2012; Yokoyama et al., 2014; Lee et al., 2015; Lewis et al., 2015), administered in doses that provide 2-4 g/day of elemental iron. It has been shown to ameliorate secondary hyperparathyroidism and vascular calcification in CKD (Iida et al., 2013; Block et al., 2015). Ferric citrate has additional benefits in that it improves iron deficiency anemia by restoring iron stores. In a phase 3

randomized controlled trial of ferric citrate therapy in non-dialysis CKD patients, mean transferrin saturation and ferritin were increased by 18.4% and 170 ng/ml respectively after 16 weeks of therapy (Fishbane et al., 2017).

Iron is an essential nutrient in both microbes and mammals. Microbes acquire iron by producing siderophores, which are small molecules that chelate and internalize iron. Siderophores play a major role in microbial physiology and virulence, and can modulate inter-bacterial competition and host cellular pathways (Holden and Bachman, 2015; Wilson et al., 2016). However, it is unknown whether gut microbes would be affected by ferric citrate therapy. Studies in non-CKD rodents have demonstrated varying effects of ferrous sulfate supplementation on the gut microbiome including a decrease in the proportion of strict anaerobes (Benoni et al., 1993; Tompkins et al., 2001; Alexeev et al., 2017). Studies in CKD models to examine the impact of iron supplementation on the gut microbiome are lacking. Given the critical role of iron in microbial growth and virulence, the present study was undertaken to test the hypothesis that the large iron load administered with ferric citrate in CKD may result in significant changes in the gut microbiome. To this end the fecal microbiome was characterized in both normal and CKD rats treated with or without ferric citrate in their chow for 6 weeks.

## **Materials and Methods**

### ***Animals***

All experiments were approved by the University of California, Irvine Institutional Committee for the Use and Care of Experimental Animals. Eight-week old male Sprague-Dawley rats were purchased from Charles River Labs (Raleigh, NC). They were housed in a climate-controlled

vivarium with 12 h day/night cycles and provided access to food and water ad libitum. The CKD groups were subjected to 5/6 nephrectomy by removing the upper and lower thirds of the de-capsulated left kidney, followed by right nephrectomy 7 days later as described previously (Vaziri et al., 2007). The Normal (NL) group underwent a sham operation. General anesthesia was induced with 5% inhaled isoflurane (Piramal Clinical Care, Bethlehem, PA) and maintained at 2-4% isoflurane during surgery. For pain relief, rats were given 0.05 mg/kg of Buprenex (Reckitt Benckiser Pharmaceutical Inc., Richmond, VA, USA). The NL and CKD groups were randomly assigned to a regular diet or a diet containing 4% ferric citrate (FC) for 6 weeks. The animals were then placed in metabolic cages for a 24 h urine collection. Systolic blood pressure (SBP) was measured by tail plethysmography as described previously (Vaziri et al., 2002). Animals were euthanized by cardiac exsanguination under isoflurane anesthesia and colons were resected. Cecal and colon stool contents were collected and processed for determination of microbial community composition as described below.

### ***Blood and urine biochemistries***

Plasma phosphorus, calcium, iron, blood urea nitrogen (BUN) and urine creatinine were determined using QuantiChrom Assay Kits from BioAssay Systems (Hayward, CA). Plasma creatinine was measured using capillary electrophoresis at the O'Brien Kidney Research Core Center (UT Southwestern, Dallas, TX). Blood hemoglobin was determined using the AimStrip Hb meter (Ermarine Laboratories Inc., San Antonio, TX).

### ***Mass Spectrometry for indoxyl sulfate and p-cresyl sulfate***

Analysis was done at the Mass Spectrometry facility at University of California, Irvine's Chemistry department. We used a modified protocol based on previously described methods (Shu et al., 2016; Kanemitsu et al., 2017). A 0.1 mL aliquot of plasma was treated with 200  $\mu$ L of acetonitrile for protein precipitation containing 2  $\mu$ g/ml of hydrochlorothiazide.

Hydrochlorothiazide was used as the internal standard. The mixture was vortexed and homogenize for 10 min in a water bath sonicator then centrifuged at 16,400 g for 15 min at 4°C. The supernatant was collected into a 2 ml microcentrifuge tube and evaporated to dryness at 60°C. The dried extract was reconstituted with 100  $\mu$ L of 25% acetonitrile.

Indoxyl sulfate, *p*-cresyl sulfate and hydrochlorothiazide reference standards were purchased from Fisher Scientific (catalog# 501151154, AAA1707901 and 5001437615). A stock 2 mg/ml solution was prepared with water and dilutions were prepared with 25% acetonitrile with 4  $\mu$ g/ml hydrochlorothiazide (internal standard) to generate a standard curve ranging 1-2000 ng/ml. Standards and prepared samples were injected (10  $\mu$ L) into the HPLC-MS/MS instrument, Waters Quattro Premier XE equipped with UPLC. The UPLC has a BEH C18 column which allows rapid sample throughput. Mobile phase A was water with 5 mM ammonium formate and mobile phase B was 95% methanol with 5 mM ammonium formate. Analysis was performed in negative ionization mode by using multiple reaction monitoring MS/MS with standard calibration. The transition (*m/z*) values were indoxyl sulfate 211.97>80.36, *p*-cresyl sulfate 186.94>107.30, hydrochlorothiazide 296.96>270.08.

### ***Microbial DNA extraction and 16S rRNA gene amplicon sequencing***



Cecal and fecal samples stored at -80°C were sent to the CGEB-Integrated Microbiome Resource (IMR) at Dalhousie University for extraction, library preparation and sequencing of the 16S V6-V8 region on an Illumina MiSeq. Microbial DNA was extracted with a MO BIO PowerFecal extraction kit, and amplification and sequencing was done as previously described (Comeau et al., 2017) (see also IMR website <http://cgeb-imr.ca/protocols.html>).

### ***Sequence analysis***

A total of 32,000 sequences per sample were retained following QC filtering and rarefaction, and these remaining sequences were clustered into 97% Operational Taxonomic Units (OTUs) using open-reference clustering. All steps were conducted with Microbiome Helper, as described previously<sup>39</sup>. The resulting OTU table was then used for further analysis within the Vegan package in R (Oksanen et al., 2007). Alpha- and beta-diversity metrics were calculated, and data were also analyzed using STAMP (Parks et al., 2014). **Alpha-diversity metrics assess both the richness (number of taxa) and evenness (distribution of species abundances). We included three metrics of alpha diversity that each combine richness and evenness: Shannon, Simpson (this metric gives more weight to dominant species) and Fisher analyses as implemented in the Vegan package in R (Oksanen et al., 2007).**

### ***Statistical Analysis***

Data was screened for outliers using the Grubbs' test (extreme studentized deviate method, <http://graphpad.com/quickcalcs/grubbs1/>). Group data was analyzed using one-way ANOVA with posthoc Tukey, and  $p < 0.05$  was considered significant. A non-parametric statistical test was applied to verify the clustering of microbial diversity. Permutational Multivariate Analysis of

Variance (PERMANOVA) was applied to determine which factors (i.e. being in the CKD group or ferric citrate treatment) explained the most variation in microbial community composition.

## Results

### *General data*

Data are shown in **Table 1** for the 4 study groups: NL (n=5), NL+FC (n=6), CKD (n=4) and CKD+FC (n=6). Compared to the CKD rats consuming regular chow, the CKD rats consuming a diet supplemented with 4% ferric citrate showed a significant increase in plasma iron and blood hemoglobin concentration, a modest decline in arterial pressure and plasma creatinine concentration, a modest rise in creatinine clearance, but no significant change in blood urea concentration. Plasma phosphorus levels were decreased as expected, as ferric citrate is a dietary phosphate binder.

Plasma levels of the gut-derived uremic toxins indoxyl sulfate and *p*-cresyl sulfate were increased in CKD animals compared to NL sham controls, but were not significantly different between the CKD groups. Indoxyl sulfate was  $594 \pm 69$  vs.  $2253 \pm 328$  vs.  $2235 \pm 226$  ng/ml in NL vs. CKD vs. CKD+FC groups (mean  $\pm$  SEM, ANOVA  $P < 0.01$  with posthoc Tukey  $P < 0.05$  between NL vs. CKD and NL vs. CKD+FC). The *p*-cresyl sulfate levels were  $113 \pm 51$  vs.  $450 \pm 150$  vs.  $491 \pm 100$  ng/ml in NL vs. CKD vs. CKD+FC groups (ANOVA  $P = 0.05$  and nonsignificant on pairwise comparisons).

### *Gut microbial communities in CKD rats are less diverse*

A total of 42 fecal and cecal samples from the 4-6 animals in each of the four treatment groups were processed for microbiome analysis. DNA was extracted and then the V6-V8 region of the 16S rRNA gene was amplified and sequenced as described in the Materials and Methods. Distributions of taxa per phyla and highest level of resolution achievable are shown in **Figure 1**; the phyla Firmicutes and Bacteroides comprised the majority of all communities. CKD rats (treated and untreated) had higher levels of Bacteroidetes and fewer Firmicutes compared to control rats ( $p < 0.01$  across groups), which had an inverse Bacteroidetes/Firmicutes ratio (**Figure 1A** and **Supplemental Figure 1**). **Figure 1B** shows the distribution of taxa identified at the most resolved level of taxonomy, usually genus, but sometimes order or family. For example, the second most abundant OTU was an uncultured Bacteroides (f\_S24-7 as shown in **Figure 1B**), a family that is often found at high levels in the guts of homeothermic mammals (Ormerod et al., 2016).

Rarefaction curves, displaying the number of bacterial types as a function of the number of sequence reads that were sampled, are shown in **Figure 2** and **Supplemental Figure 2** and suggest that the CKD rats, and especially the untreated control CKD rats, had lower and more variable gut microbial diversity. The diversity metrics displayed in **Figure 3** also demonstrate the variability of the CKD rats, with some samples displaying significantly less diversity. Analysis of fecal samples showed a mean Fisher's alpha of 177 vs. 174 in NL vs. NL+FC, and 114 vs. 141 in CKD vs. CKD+FC ( $P < 0.05$  across groups with significant differences between NL vs. CKD and NL+FC vs. CKD). Alpha-diversity was decreased 36% in the CKD group compared to NL animals, and was decreased less in the CKD+FC group (20% less diversity compared to NL). Cecal samples were not significantly different across groups ( $P = 0.3$ ) though there was a similar

trend for decreased diversity in the CKD groups (Fisher's alpha decreased 20% and 10% in CKD and CKD+FC respectively, compared to NL animals).

A Bray-Curtis distance matrix is displayed as a non-metric multidimensional scaling ordination plot in **Figure 4**. In these types of plots, samples with more similar microbial community composition are located closer to each other on the plot. This plot suggests that the microbial communities of individual NL rats were more similar to each other in composition than those from the CKD rats, as shown by the greater spread of the CKD-derived samples in the NMDS plot (**Figure 4**).

#### ***Ferric citrate treatment affects gut microbial composition in both normal and CKD rats***

Ferric citrate treatment in both NL and CKD animals shifted the samples toward the origin of the vertical axis, NMDS2 (**Figure 4**), so that they were clustered together more closely, with less widespread individual variability. Treatment with ferric citrate increased diversity in CKD rats, almost to the levels observed in normal NL rats (**Figure 3**), and reduced abundances of Firmicutes in both normal and CKD rats (**Figure 1A** and **Supplemental Figure 1**). The Verrucomicrobia, which were largely *Akkermansia muciniphila*, had elevated abundances in the non-treated CKD rats which increased further with ferric citrate treatment in both the Normal and CKD rat groups (**Figure 5**). *A. muciniphila* increased from near zero to 12% of the community in the treated NL rats (**Figure 5**). Similarly, other tryptophanase-possessing families, the Clostridiaceae and Enterobacteriaceae, were increased by ferric citrate treatment in both NL and CKD animals (data not shown).

A non-parametric statistical test was applied to verify the groupings that can be seen in **Figure 4**. Permutational Multivariate Analysis of Variance (PERMANOVA) is a multivariate analysis of variance which tests the null hypothesis that there are no differences in microbial community composition between the health status or treatment groups, and was applied to determine which variables explained the most variation in microbial community composition. The control vs CKD groupings explained 9% ( $p < 0.01$ ) of the variance in the samples (**Supplemental Table 1**), while FC-treatment explained about 11% of the variance ( $p < 0.01$ ). Samples from the colonic feces or cecum had similar composition (**Figure 1**) and clustered near one another (**Figure 4**), suggesting that fecal samples were largely representative of cecal samples. However, the PERMANOVA result shows that there was a significant difference between the fecal and cecal samples, with a small effect size, explaining less than 5% of the variance (**Supplemental Table 1**).

## Discussion

Our study supports prior reports (Aronov et al., 2011; Vaziri et al., 2013a; Wong et al., 2014) showing that the gut microbiome is markedly altered in CKD. CKD rats had lower and more variable gut microbial diversity. Both treated and untreated CKD rat microbial communities contained higher levels of Bacteroidetes and lower levels of Firmicutes, in contrast to Normal animals that have more Firmicutes. Ferric citrate treatment in CKD rats increased diversity almost to the levels observed in normal animals and also brought the microbial community compositions across samples closer together. Thus the “Anna Karenina hypothesis” may apply to the uremic microbiome, where “all happy families look alike; each unhappy family is unhappy in its own way” (Zaneveld et al., 2017). Ferric citrate treatment also increased levels of

tryptophanase-possessing families that are associated with production of indole and *p*-cresyl uremic toxins (Verrucomicrobia, Clostridiaceae and Enterobacteriaceae) however measured plasma levels of indoxyl sulfate and *p*-cresyl sulfate were not significantly different with FC treatment. The gut microbial changes were associated with improved kidney function (increased creatinine clearance, lower plasma creatinine) and decreased hypertension in the ferric citrate-treated CKD animals.

In an earlier study in hemodialysis patients, phylogenic microarray analysis of microbial DNA demonstrated highly significant differences in the abundance of over 200 bacterial OTUs belonging to 23 bacterial families compared to healthy controls (Vaziri et al., 2013a). The OTUs that were markedly increased included the Cellulomonadaceae, Clostridiaceae, Enterobacteriaceae, Moraxellaceae, Pseudomonadaceae and Verrucomicrobiaceae families (Vaziri et al., 2013a). The Clostridiaceae, Enterobacteriaceae and Verrucomicrobiaceae are of particular interest as these microbes possess indole and *p*-cresyl-forming enzymes (i.e. tryptophanase-possessing families) (Wong et al., 2014) and generate gut-derived uremic toxins such as indoxyl sulfate and *p*-cresyl sulfate that translocate back into the bloodstream and contribute to systemic inflammation (Mafra et al., 2014; Rossi et al., 2014; Lau et al., 2015).

In the current animal study, ferric citrate therapy increased Clostridiaceae, Enterobacteriaceae and Verrucomicrobiaceae in the stool from CKD animals however plasma levels of the tryptophan-derived uremic toxins indoxyl sulfate and *p*-cresyl sulfate were not significantly increased. Ferric citrate therapy was associated with improved kidney function, suggesting that a potentially deleterious increase in production of gut-derived uremic toxins was offset by

improved urinary clearance. Another pathway of interest may have been the increased abundance of *A. muciniphila* (Verrucomicrobiaceae). *A. muciniphila* is a mucin-consuming bacterium that may have an important role in maintaining integrity of the intestinal mucosal barrier and has anti-inflammatory properties. This species has been suggested as a biomarker of a healthy gut status (Fujio-Vejar et al., 2017) and may be induced by a low-fiber diet; however, it does not occur outside of the Western world (Desai et al., 2016; Cani and de Vos, 2017; Ottman et al., 2017; Smits et al., 2017). The increase in *Akkermansia*, akin to a reduction in dietary fiber, is interesting and raises further questions about whether ferric citrate influences gut mucins. *Akkermansia* metabolizes mucin to acetate and propionate, short-chain fatty acids which are nutrients for the host's enterocytes (Derrien et al., 2004; Derrien et al., 2011), and creates a positive feedback loop that stimulates mucin secretion (Derrien et al., 2010). *A. muciniphila* has been shown to decrease endotoxemia and regulate adipose tissue metabolism and glucose homeostasis (Everard et al., 2013; Shin et al., 2014; Anhê et al., 2015).

ESKD patients show decreased numbers of gut bacteria such as Lactobacillaceae and Prevotellaceae that are able to produce the short-chain fatty acid butyrate, also an important nutrient source for host enterocytes (Vaziri et al., 2013a; Wong et al., 2014). It was recently proposed that the use of oral iron supplements might further contribute to gut microbiome alterations (Kortman et al., 2017), extrapolating from studies in African children where supplemental iron decreased the abundances of bacteria considered to be beneficial, such as Bifidobacteriaceae and Lactobacillaceae (Jaeggi et al., 2015), and increased gut permeability (Nchito et al., 2006). In an *in vitro* model of the human colon, where intestinal epithelial Caco-2 cells were inoculated with human microbiota, incubation with ferrous sulfate or ferric citrate

altered the microbiome population and also decreased levels of Bifidobacteriaceae and Lactobacillaceae (Kortman et al., 2015). The investigators noted cytotoxicity to Caco-2 cells with effluent from iron-treatment conditions that contained microbe-derived metabolites (Kortman et al., 2015). The present study confirmed a decrease in *Lactobacillus* counts in both Normal and CKD rats treated with ferric citrate; however, this did not translate to worse kidney function or blood pressure outcomes. It should be noted that, unlike the soluble ferrous sulfate and ferric citrate compounds used in the above studies, the ferric citrate employed in our study is an extremely large and insoluble complex which is used as a phosphate binder.

Ferric citrate has other beneficial effects that may have overwhelmed any systemic impact from the altered gut microbiome. As a phosphate binder, ferric citrate ameliorates hyperphosphatemia, secondary hyperparathyroidism and vascular calcification (Yokoyama et al., 2012; Iida et al., 2013; Yokoyama et al., 2014; Block et al., 2015; Lee et al., 2015; Lewis et al., 2015). Further, ferric citrate improves iron deficiency anemia (Fishbane et al., 2017) as evidenced by the increased hemoglobin levels in the treated CKD rats.

The current study is not able to define to what extent the microbiome alterations contributed to overall systemic benefits, as opposed to direct effects of ferric citrate itself. Future investigations with germ-free rodents would be one way to separate the impact of ferric citrate on the host alone, independent of the microbiota. Further, there is the emerging concept of the microgenderome whereby sex differences in the microbiome may influence systemic outcomes (Markle et al., 2013; Flak et al., 2013; Elderman et al., 2018); the current ferric citrate



investigation will need to be replicated in female CKD rats to delineate sex differences in measured outcomes.

In summary, our study suggests that CKD is associated with lower and more variable gut microbial diversity. Ferric citrate therapy decreased hyperphosphatemia, improved anemia, and improved gut microbial diversity almost to the levels observed in Normal animals.

Lactobacillaceae were further decreased with ferric citrate therapy, whereas Verrucomicrobiaceae increased. Of particular interest within the Verrucomicrobiaceae is *A. muciniphila*, which has anti-inflammatory properties and promotes integrity of the intestinal barrier. The gut-derived uremic toxins indoxyl sulfate and p-cresyl sulfate were not significantly altered with ferric citrate therapy. The CKD rats treated with ferric citrate had less hypertension and better kidney function as assessed by plasma creatinine and urinary creatinine clearance. Overall, our findings support a beneficial impact of oral ferric citrate in CKD in terms of promoting gut microbial diversity and improved kidney function.

Acknowledgments: WLL has received funding from the American Heart Association, Hub Therapeutics, Sanofi and UC Irvine Division of Nephrology. NDV has received funding from Reata, Keryx Biopharmaceuticals and Novartis. AN received support from a Brazil Science Without Borders Grant. YS received support from the Sumitomo Life Welfare and Culture Foundation and Overseas Research Scholarship, and Fukuoka University School of Medicine Alumni Overseas Research Scholarship.

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*Contributed new reagents or analytic tools:* Langille, Whiteson

*Performed data analysis:* Lau, Vaziri, Nunes, England, Whiteson

*Wrote or contributed to the writing of the manuscript:* Lau, Vaziri, Nunes, Comeau, Langille, England, Whiteson.

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## Footnotes

This work was supported by an unrestricted research grant from Keryx Biopharmaceuticals Inc., Boston, MA.



## Legends for Figures

**Figure 1.** Relative abundance of bacterial taxa at the phylum level (A) and the highest level of resolution achievable (B) from 16S rRNA gene sequencing and 97% Operational Taxonomic Unit (OTU) clusters. Fecal and cecal samples were from untreated and ferric citrate (FC) treated Normal (NL) and Chronic Kidney Disease (CKD) rats. The 2<sup>nd</sup> most abundant taxon at the genus level is an uncultured Bacteroidetes (f\_ S24-7) that is often found in the gut communities of homeothermic mammals. g = genus, f = family, o = order.

**Figure 2.** Rarefaction curve showing the number of 97% identity bacterial Operational Taxonomic Units (OTUs) from fecal samples in the four study groups with/without ferric citrate (FC) therapy. The x-axis shows the number of sequences sampled to calculate the corresponding “OTUs Observed” value for the y-axis. NL = normal controls, CKD = chronic kidney disease animals, +FC = Ferric Citrate treated.

**Figure 3.** Alpha-diversity metrics of rat fecal and cecal samples from untreated and ferric citrate (FC) treated Normal controls and CKD rats. Three alpha-diversity metrics are shown: Shannon (top panel), Simpson (middle panel), and Fisher (bottom panel). NL = normal controls, CKD = chronic kidney disease animals, +FC = Ferric Citrate treated.

**Figure 4.** Non-Metric Multidimensional Scaling (NMDS) ordination plot of Bray-Curtis distances from 16S rRNA gene sequences is shown for each sample, represented by treatment group and sample type. NL = normal controls, CKD = chronic kidney disease animals, +FC = Ferric Citrate treated.

**Figure 5.** Abundances of bacterial species amplicons that differed across the treatment groups. A) *Akkermansia* spp. B) *Lactobacillus* spp. Significance testing was done with the Tukey-

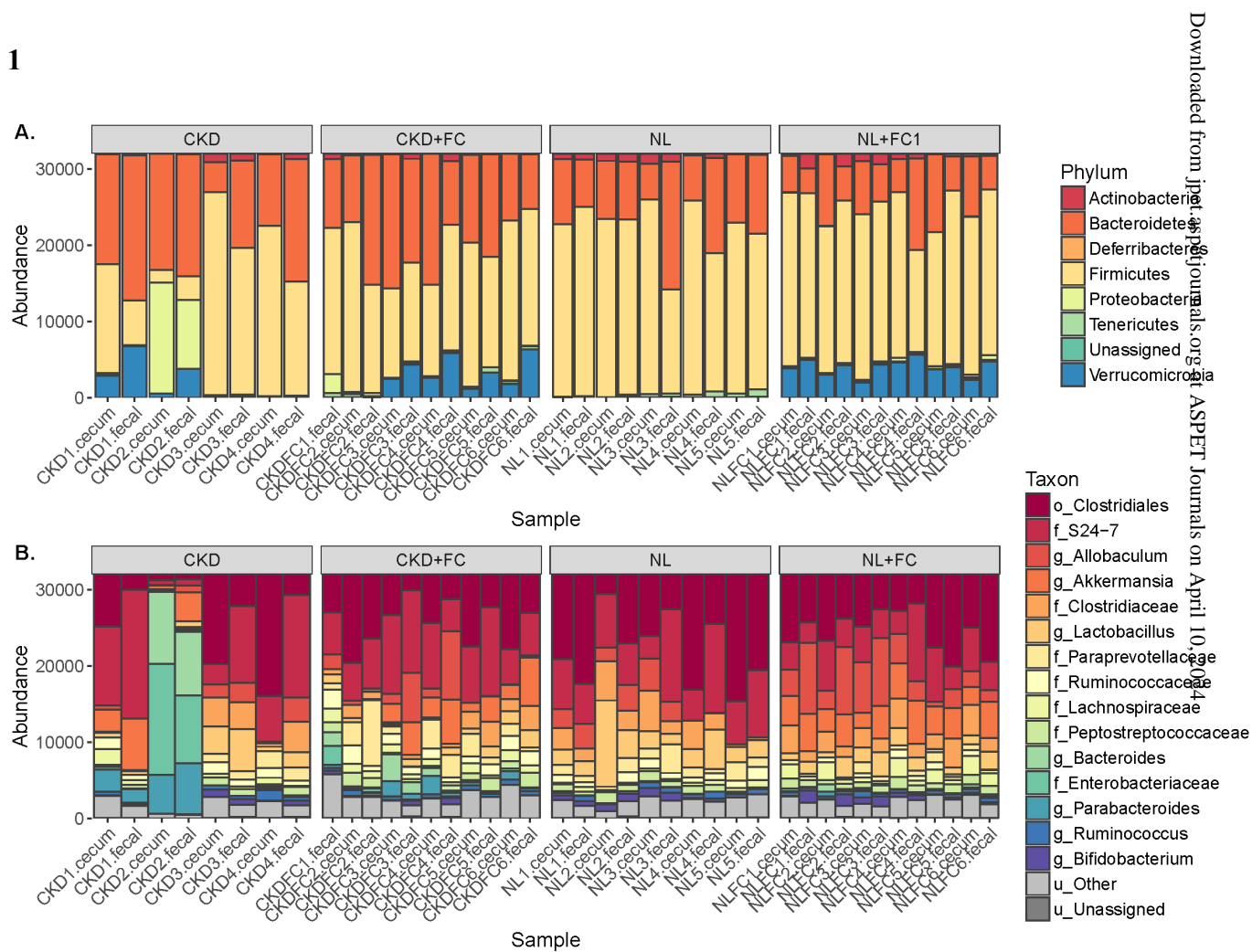
Kramer post-hoc test in STAMP (see Methods). CKD = chronic kidney disease animals,  
plusFeCitrate = Ferric Citrate treated.

**Table 1.** Body weight, tail blood pressure, and plasma and urine data in the 4 study groups.

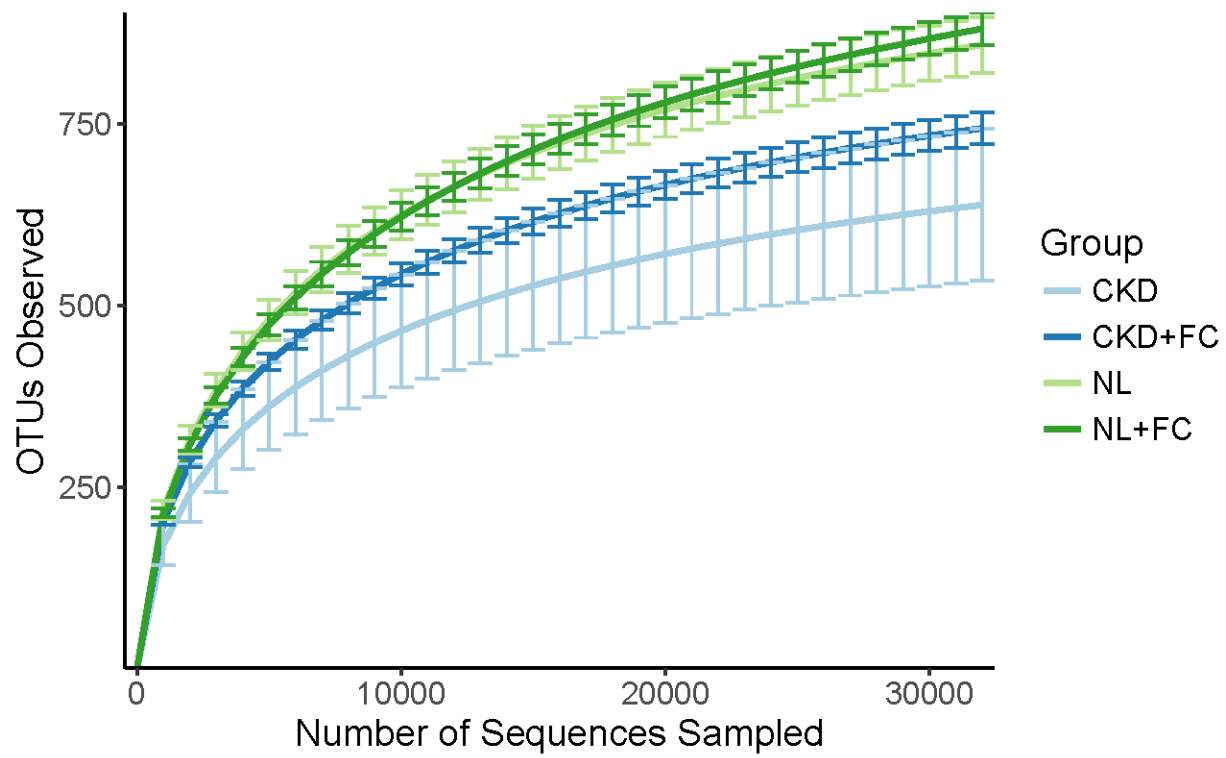
Variables	Normal rats (NL)		5/6 nephrectomy CKD	
	NL	NL+FC	CKD	CKD+FC
Systolic blood pressure (mmHg)	108.7±3.1	111.7±1.1	149.0±3.4 <sup>*†</sup>	112.8±4.8 <sup>‡</sup>
Body weight Week 6 (g)	408.3±12.8	387.8±8.4	290.8±18.3 <sup>*†</sup>	357.8±15.5 <sup>*‡</sup>
Plasma phosphorus (mg/dl)	8.5±0.2	8.7±0.2	12.2±0.2 <sup>*†</sup>	8.7±0.6 <sup>‡</sup>
Plasma calcium (mg/dl)	9.8±0.7	10.4±0.4	9.0±0.5	9.8±0.3
Plasma total iron (µg/dl)	218.4±20.4	285.0±19.1	167.1±7.5 <sup>†</sup>	196.2±21.8 <sup>†</sup>
Hemoglobin (g/dl)	14.9±0.1	14.8±0.1	8.6±0.8 <sup>*†</sup>	12.4±0.4 <sup>*†‡</sup>
BUN (mg/dl)	20.1±0.5	20.4±0.3	160.9±25.9 <sup>*†</sup>	93.6±26.2 <sup>†</sup>
Plasma creatinine (mg/dl)	0.2±0.01	0.2±0.03	2.6±0.2 <sup>*†</sup>	1.1±0.2 <sup>*†‡</sup>
Creatinine clearance (ml/min*kg)	10.3±2.0	12.1±1.1	0.8±0.1 <sup>*†</sup>	2.5±0.3 <sup>*†</sup>

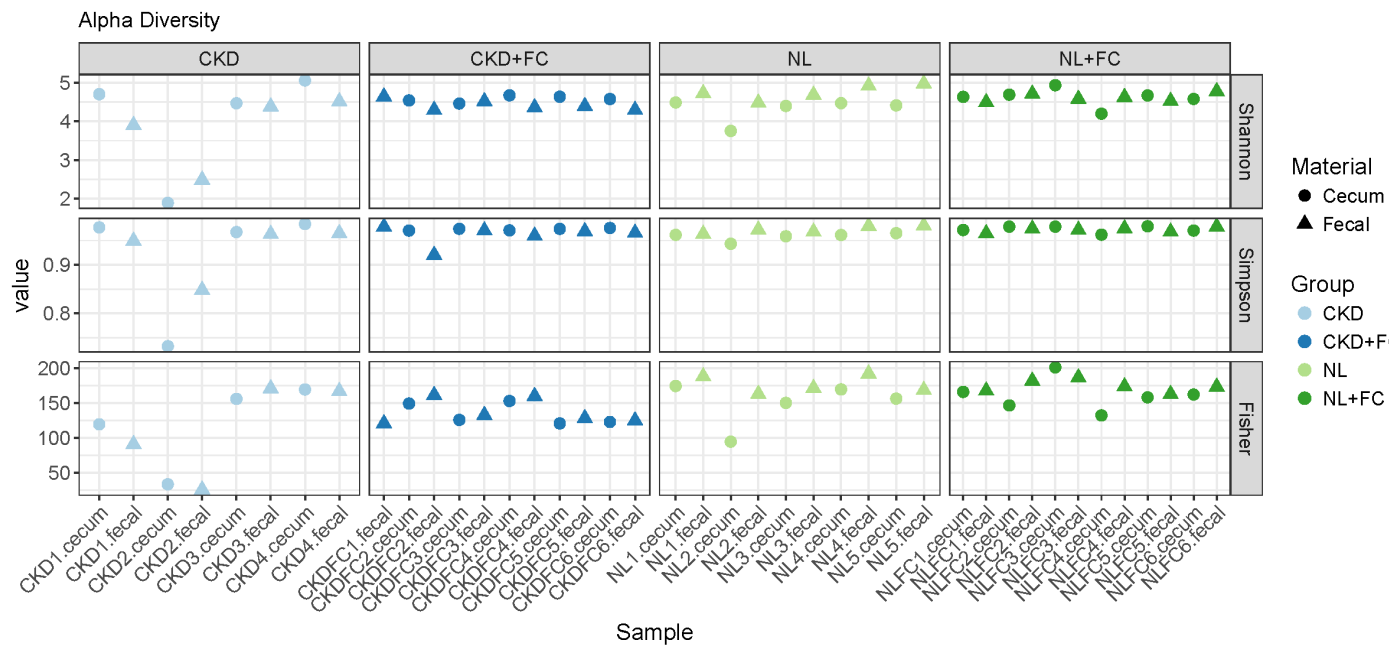
Abbreviations: BUN, blood urea nitrogen; CKD, chronic kidney disease; NT-pro BNP, N-terminal pro brain natriuretic peptide; FC: 4% ferric citrate in diet; FGF-23, fibroblast growth factor 23. Data are mean ± SEM. n=4-6 per group, \* p<0.05 vs. NL, † p<0.05 vs. NL+FC, ‡ p<0.05 vs. CKD

Figure 1

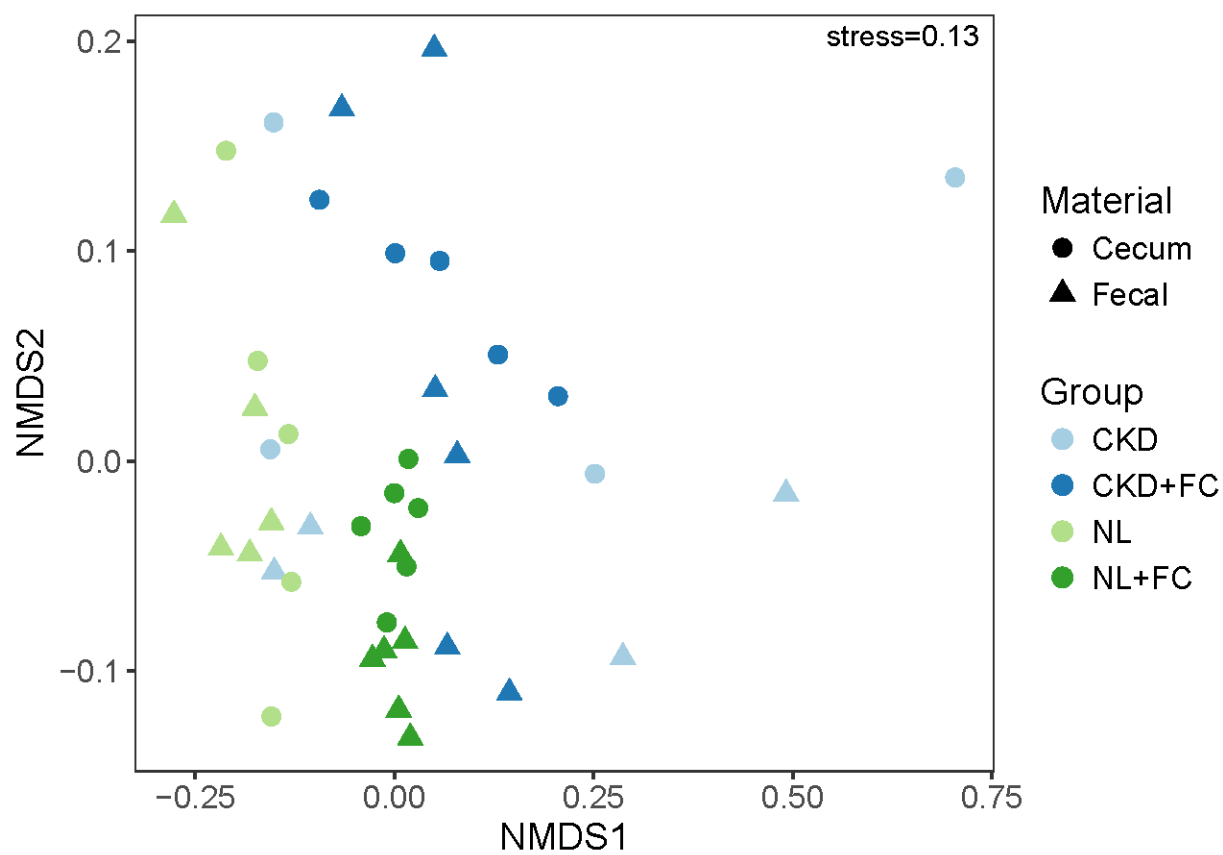


**Figure 2**



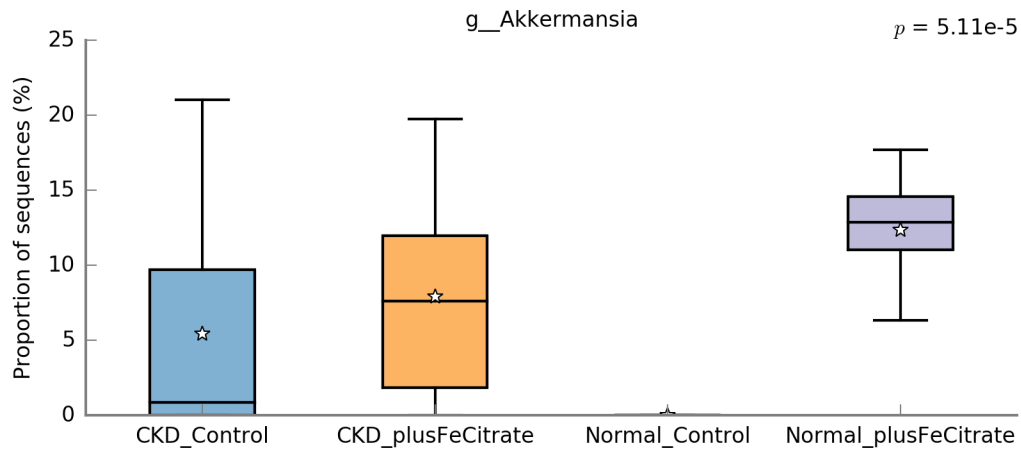
**Figure 3**

**Figure 4**

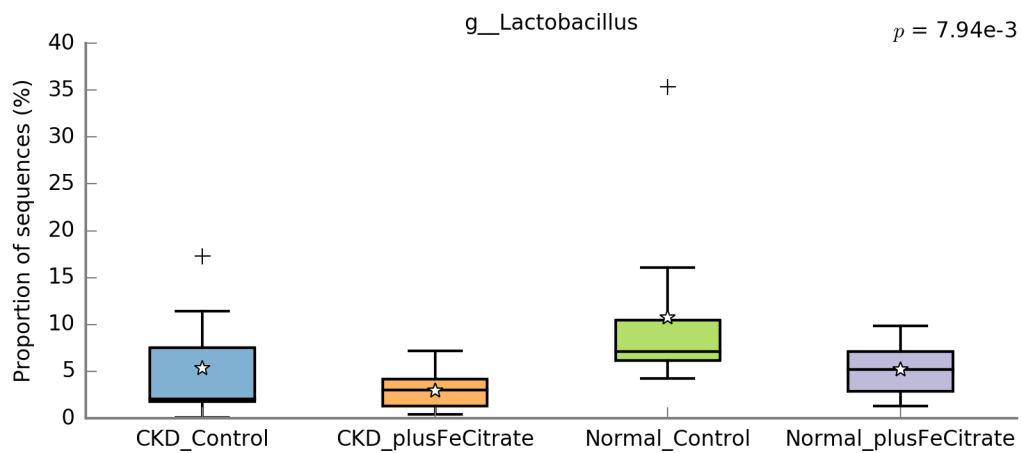


**Figure 5**

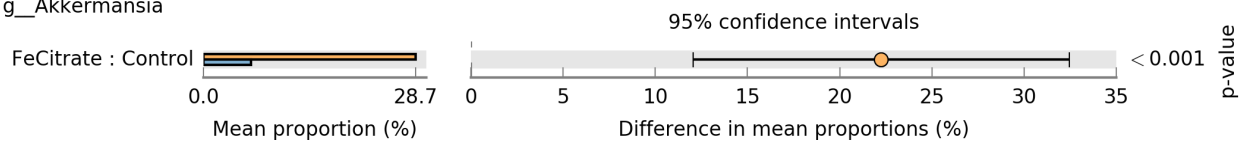
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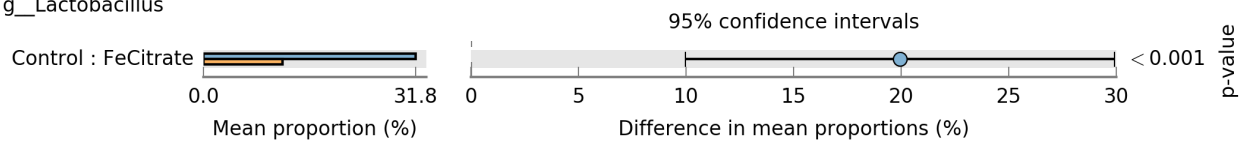
B.



**g\_Akkermansia**



**g\_Lactobacillus**





## **The Journal of Pharmacology and Experimental Therapeutics**

### **Supplemental Data**

**Article Title: The phosphate binder ferric citrate alters the gut microbiome in rats with chronic kidney disease**

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Division of Nephrology and Hypertension, Department of Medicine, University of California, Irvine, CA, USA (W.L.L., N.D.V., A.C.F.N., M.K.)

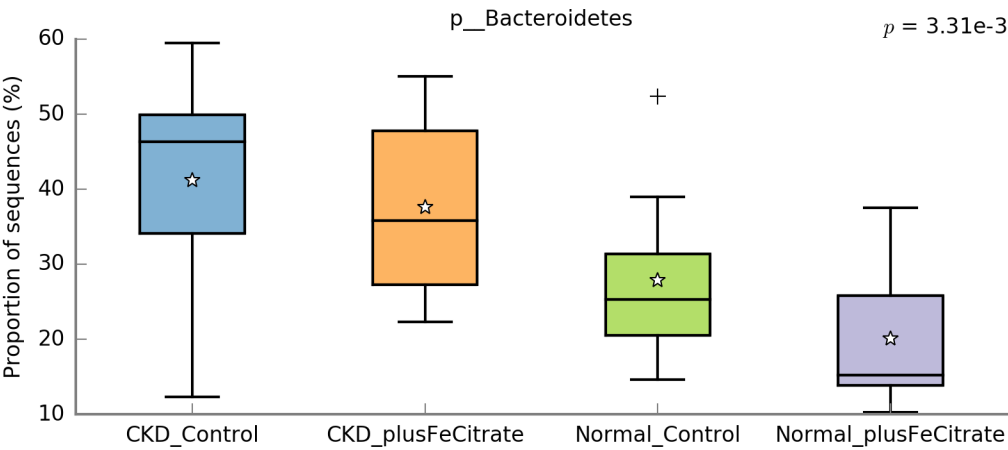
CGEB-Integrated Microbiome Resource (IMR) and Department of Pharmacology, Dalhousie University, Halifax, NS, Canada (A.M.C., M.G.I.L.)

Department of Molecular Biology & Biochemistry, University of California, Irvine, CA, USA (W.E., J.P., K.W.)

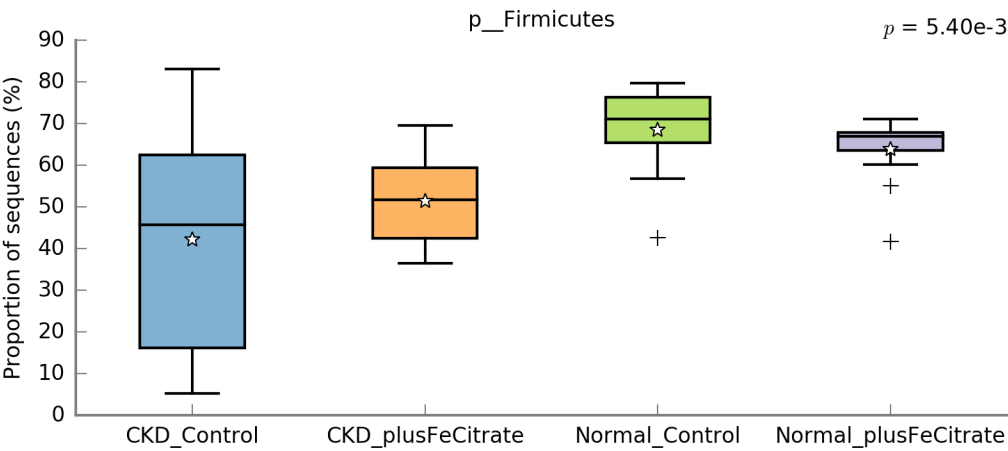
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Supplemental Figures

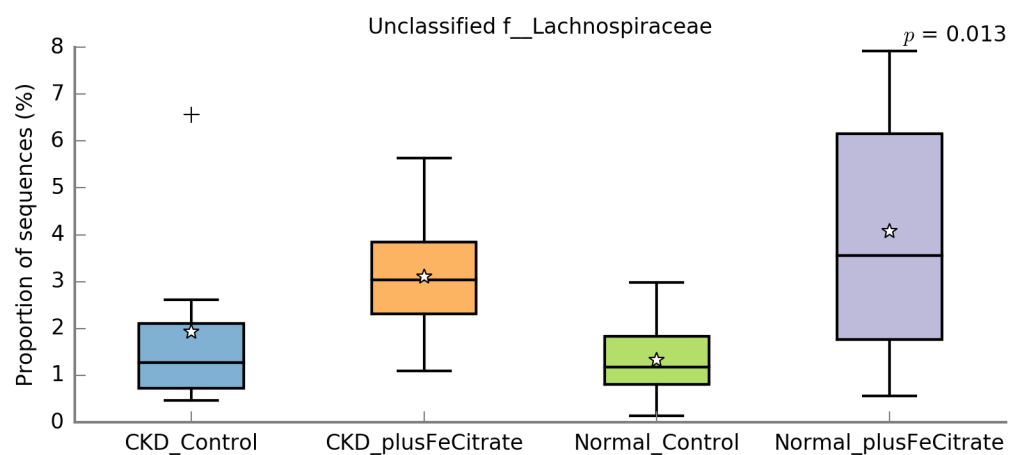
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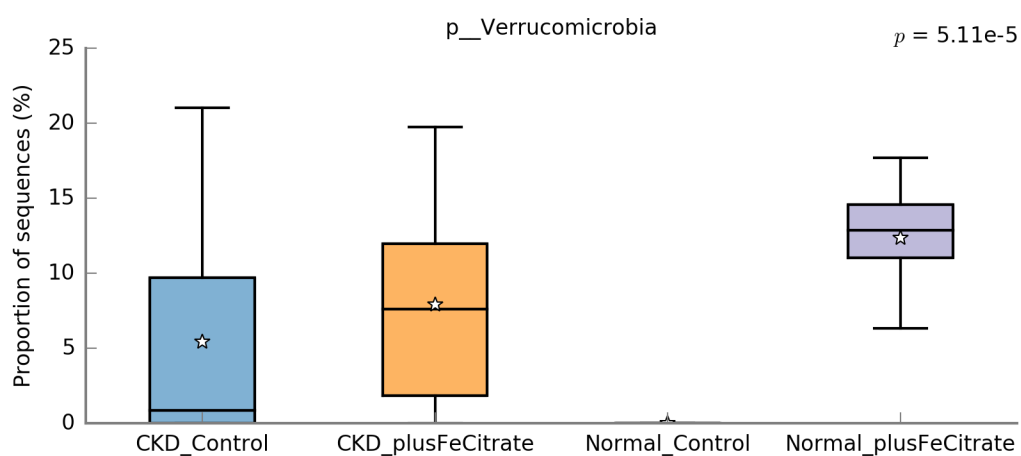
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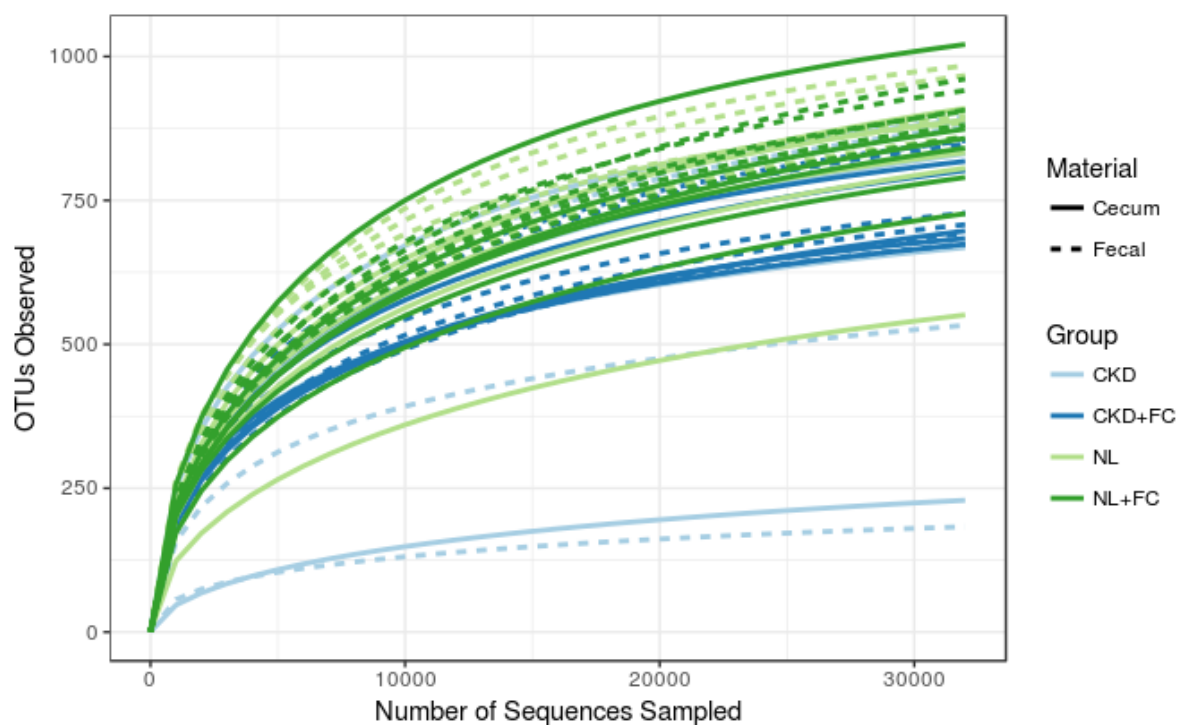
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D.



**Supplemental Figure 1.** Abundances of several bacterial taxa of interest across the four treatment groups. A) Bacteroidetes at the phyla level, B) Firmicutes at the phyla level, C) Lachnospiraceae at the family level and D) Verrucomicrobia abundance at the phyla level, largely comprised of *A. muciniphila*. CKD = chronic kidney disease animals



**Supplemental Figure 2.** Rarefaction curves of the 97% OTUs based on 16S rRNA gene sequences showing the number of OTUs in each sample. NL = normal controls, CKD = chronic kidney disease animals, +FC = Ferric Citrate treated.

**Supplemental Table 1.** Permanova test of the similarity of the microbial communities in the sampling groups, implemented with the Adonis test in the Vegan package in R. CKD = chronic kidney disease animals

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
CKD vs Normal	1	0.7748	0.77476	4.6254	0.08957	0.01**
Cecal vs fecal	1	0.3626	0.36256	2.1646	0.04192	0.04*
Ferric citrate treatment	1	0.9843	0.98434	5.8767	0.11380	0.01**
Disease and Treatment	1	0.4980	0.49795	2.9729	0.05757	0.01**
Residuals	36	6.0300	0.16750	0.69714		
Total	40	8.6496	1.00000			

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Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

Permanova Call:

```
adonis(formula = indata_sorted ~ DiseaseState + Material + Treatment + D.T, data = inmeta_sorted,
permutations = 99)
```

Permutation: free

Number of permutations: 99