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METABOLIC PROFILING OF A CHRONIC KIDNEY DISEASE COHORT REVEALS METABOLIC PHENOTYPE MORE LIKELY TO BENEFIT FROM A PROBIOTIC

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ABSTRACT: ***Persistent reduction in Glomerular Filtration Rate (GFR) is a hallmark of Chronic Kidney Disease (CKD) and is associated with an elevation of Blood Urea Nitrogen (BUN). This metabolomics pilot study sought to identify metabolites that differentiated patients with CKD whose BUN decreased on a probiotic and possible associated mechanisms. Metabolomics was used to analyze baseline plasma samples from subjects previously diagnosed with CKD Stage III-IV. Patients had participated in a dose escalation study of the probiotic Renadyl™. A total of 24 samples were categorized depending on whether BUN increased or decreased from baseline after 4 months of probiotic use. Multivariate analysis was used to analyze the data and determine the metabolites that best differentiated the phenotypic groups. The sixteen patients who had a decrease in BUN were not significantly different based on demographic and clinical measures from those whose BUN increased or did not change with the exception of age. Eleven of the fourteen metabolites that differentiated the groups were known to be modulated by gut microflora, which may eventually provide a mechanistic link between probiotic and outcomes. Metabolomics revealed metabolites at baseline that may predict individuals with CKD that would most benefit from probiotics.***

KEY WORDS: BUN, Chronic kidney disease, Microbial metabolism, Multivariate analysis, NMR Metabolomics, Probiotics

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Abbreviations Used: AKI: Acute Kidney Injury, BMI: Body Mass Index, BUN: Blood Urea Nitrogen, CFU: Colony Forming Units, CKD: Chronic Kidney Disease, CPMG: Carr-Purcell-Meiboom-Gill sequence, CRP: C-reactive Protein, ESRD: End Stage Renal Disease, GFR: Glomerular Filtration Rate, IQR: Inter Quartile Range, NMR: Nuclear Magnetic Resonance Spectroscopy, OPLS-DA: Orthogonal partial least squares discriminant analysis, PCA: Principal component analysis, SAS: Statistical Analysis System, TMAO: Trimethylamine N-oxide, VIP: variable importance for projections.

INTRODUCTION

Chronic Kidney Disease (CKD) has become the 9th leading cause of death in the United States (Johnson et al., 2014). More than 20 million people, aged 20 years or older in the United States have CKD (Shahinian et al., 2013). The population of people with far advanced CKD, also called End Stage Renal Disease (ESRD), on dialysis in 2013 was 661,648 (Saran et al., 2015). Most recently, it has been suggested that the prevalence of CKD in adults will increase to 14.4% by 2020 and 16.7% by 2030 (Hoerger et al.). Earlier detection and awareness by providers and the associations of CKD with advanced age, diabetes, hypertension, cardiovascular disease and obesity (Tanner et al., 2012) have all been suggested to explain the changing incidence of CKD worldwide. Providing renal replacement therapies such as dialysis to patients with ESRD is lifesaving but is very expensive and challenging. New treatment approaches capable of preventing or delaying progression to ESRD would not only reduce these costs, but improve patient quality of life.

CKD is usually identified in the clinic with an elevation of Blood Urea Nitrogen (BUN) or serum creatinine and is

defined as a reduction in Glomerular Filtration Rate (GFR) of < 60 ml/min/1.73² BSA for 3 months or more. This definition includes only its filtering ability and omits other kidney functions such as endocrine and tubular secretory functions which might also change as the kidney begins to lose its filtering capacity. The metabolome could reflect changes in each of these functions; however, the interaction between kidney pathophysiology and the metabolome is not fully investigated (Rhee, 2015). Recently, metabolomics has been used to examine changes in metabolite profiles in patients with AKI before and after Ischemia/Reperfusion (Wei et al., 2014) and applied to identify metabolites that would predict the kidney rejection after transplantation in children (Blydt-Hansen et al., 2014). These findings have begun to provide biomarkers and mechanistic insights into early disease pathogenesis.

The intestinal epithelial barrier facilitates cross-talk between the gut microbiome and kidney (Ramezani and Raj, 2014). Nutrients and other exogenous materials are processed by the gut microbiome and enter the bloodstream through this barrier, and conversely components of the blood not removed by the kidney can enter the intestinal lumen and influence the health and composition of the microbiome. Progression of CKD is, for example, associated with increased microbial counts in both the duodenum and jejunum (Strid et al., 2003) and low levels of *lactobacilli, bifidobacteria, and prevotellacae* (Gibson and Roberfroid, 1995;Schepers et al., 2010;Vaziri et al., 2013). A rise in luminal concentrations of urea (Kang, 1993) and uric acid (Vaziri et al., 1995), among other metabolites (Meinardi et al., 2013), are thought to contribute to this shift in population, while production of uremic toxins such as indoxyl sulfate (Meijers and Evenepoel, 2011), p-cresol sulfate (Liabeuf et al., 2010) and TMAO (Tang et al., 2015) by colonic bacteria likely exacerbate CKD progression. End products of intestinal fermentation acetate, proprionate and butyrate, as well as probiotic treatment with acetate-producing bacteria, were recently demonstrated to protect the kidney in a model of ischemia reperfusion injury (Andrade-Oliveira et al., 2015). Prebiotics can also reduce production of uremic toxins and increase fermentation in the colon (Sirich et al., 2014). By targeting treatments to the gut microflora, the opportunity exists to simultaneously improve gut function and remove circulating uremic toxins that influence progression of CKD.

Using BUN as a phenotypic marker of disease status, this exploratory study focused on understanding biomarkers and biochemical pathways involved in probiotic response in CKD Stage III-IV patients.

MATERIALS & METHODS

Subjects

The open label study of a probiotic supplement called Renadyl™ has been described previously (Ranganathan et al., 2013). Briefly, all subjects were age 18-75 years old, previously diagnosed with CKD Stage III–IV or had a serum creatinine > 2.5 mg/dL, were pre-ESRD, and stable at least one year. Details of inclusion and exclusion criteria are outlined in the clinical trial sponsored by Kibow Biotech (KB) at Thomas Jefferson University (TJU), Philadelphia, PA, in 2011-2012 (www.clinicaltrials.gov #NCT01450657).

Protocol

Samples submitted to NIH repository represented plasma samples from 27 patients who completed the trial. Study participants were given Renadyl™ (Kibow Biotech, Inc.), which contained 30 billion Colony Forming Units (CFU) of *S. thermophilus* KB 19, *L. acidophilus* KB 27, and *B. longum* KB 31 strains per enteric coated vegetarian gel capsule over a 4 month period. In month one, participants received 90 billion CFU per day. At each month after baseline, participants had blood drawn for hematology and biochemical testing and the dose of the probiotics was escalated by 90 billion CFU to a maximum of 270 CFU. This design aimed to confirm dose safety and tolerability and demonstrate measurable improvement in biochemical markers.

Baseline plasma samples from 27 of the 28 subjects who completed the trial were selected for metabolomics analysis. Two plasma samples were excluded due to poor water suppression during NMR data acquisition, and one was excluded from the analysis due to missing BUN measurements.

Metabolomics Analysis

Sample preparation, data acquisition, statistics, and pathway analysis were performed similarly as previously described (Beckonert et al., 2007). Each plasma sample (350 µL) was prepared by addition of a 0.9% saline solution containing 0.2% NaN_3 and 2 mM formate (chemical shift indicator) in D_2O. In addition, 50 µL of each plasma samples was pooled, mixed, divided into 3 aliquots, and prepared identically to the individual study samples. Metabolomics data were acquired for each individual study and pooled samples. ^1H NMR spectra were acquired on a Bruker Advance III 700 MHz NMR spectrometer (located at the David H. Murdock Research Institute at Kannapolis, NC, USA) using the 1D CPMG pulse sequence (cpmgpr1d). NMR spectra were pre-processed using ACD 1D NMR Processor 12.0 (ACD Labs, Toronto, CA). NMR bins (0.50-8.00 ppm) were made after excluding water (4.58-4.93 ppm) using intelligent binning width of 0.04 ppm and 50% looseness factor. Integrals of each of the bins were normalized to total integral of each of the spectrum.

Normalized binned NMR data were Pareto scaled and centered prior to multivariate analysis. Multivariate data analysis methods (e.g. principal component analysis [PCA], orthogonal partial least squares discriminant analysis [OPLS-DA]) were used to reduce the dimensionality and

to enable the visualization of the separation of the study groups (SIMCA 13, Umetrics, Umeå, Sweden). The PCA scores plots were inspected to ensure that the pooled samples were tightly clustered in the center of all of the individual study samples, a quality control method that is widely used in metabolites studies (Chan et al., 2011). Loadings plots and variable importance for projections (VIP) plots were inspected, and bins that had a VIP ≥ 1.0 with a jack-knife confidence interval that did not include 0 were determined to be important to differentiating the study groups. Chenomx NMR Suite 8.1 Professional software (Edmonton, Alberta, Canada) was used to match the signals in the identified bins to metabolites. All raw and processed analytical data and associated de-identified metadata have been uploaded to the publically accessible NIH Common Fund Metabolomics Data Repository (http://dx.doi.org/10.21228/M8FW2N).

Statistical Analyses

Statistical analyses were conducted using SAS 9.4 (SAS Institute Inc, Cary, NC). Normality was not assumed due to the small sample size; therefore, descriptive statistics for continuous variables are based on the median value of the sample distribution. Statistical tests for determining whether the median percent change was different from zero for the clinical data across all subjects was conducted using the two-sided Wilcoxon Signed Rank Test. Differences in continuous measurements of subject characteristics, clinical data, and binned NMR data by phenotypic group were tested for statistical significance using the two-sided Wilcoxon Rank Sum Test, and categorical subject characteristics by phenotypic group were tested using the two-sided Fisher's Exact Test. P-values < 0.05 were considered to be statistically significant.

TABLE 1. Percent change of clinical data across all study samples. Values reported are the median (IQR). *Two missing values for CRP percent change and one missing value for Potassium percent change. **Wilcoxon Signed Rank Test. †% Change calculation: (Month 4 – Baseline)/Baseline * 100.

Clinical Measurement*†	All Study Samples (n=24)	p-value**
BUN % Change	-10.4 (16.0)	**0.02**
Systolic Blood Pressure % Change	0.0 (13.6)	0.58
Diastolic Blood Pressure % Change	2.8 (24.0)	0.13
Creatinine % Change	-1.7 (10.8)	0.70
CRP % Change	0.0 (25.0)	0.78
Hemoglobin % Change	0.5 (10.7)	0.26
Potassium % Change	-2.6 (11.1)	0.23

Identification of BUN as phenotypic anchor

The Wilcoxon Signed Rank Test that was used to determine whether the median percent change of the clinical data across all study samples was statistically different from zero. As shown in Table 1, percent change in BUN was statistically different from 0 (p=0.02) while systolic blood pressure, diastolic blood pressure, creatinine, CRP, hemoglobin, and potassium were not statistically different from zero.

For the metabolomics analysis, subjects were divided into two phenotypic groups based on the change in BUN measurements over 4 months: BUN measurement decreased at 4 months compared to baseline (Decreased BUN) and BUN measurement did not decrease over the 4 month period (Increased BUN).

TABLE 2. Baseline Demographics. *Missing value for Race/Ethnicity, Diabetes, and Hypertension. **Percentages may not sum to 100 due to rounding. †Wilcoxon-Rank Sum Test for continuous variables and Fisher's Exact Test for categorical variables. ‡One subject was determined to be Stage V after meeting inclusion criteria and remained pre-ESRD.

Characteristic*	Decreased BUN** (n=16)	Increased BUN (n=8)	p-value†
Age, median (IQR), years	60.0 (13.0)	67.00 (10.0)	**0.045**
BMI, median (IQR), (kg/m2)	32.5 (9.7)	29.65 (5.8)	0.37
Gender			
Female	9 (56.3%)	5 (62.5%)	1.00
Male	7 (43.8%)	3 (37.5%)	
Race/Ethnicity			
Caucasian	8 (53.3%)	3 (37.5%)	0.83
African American	5 (33.3%)	4 (50.0%)	
Other	2 (13.3%)	1 (12.5%)	
Diabetes			
Yes	5 (33.3%)	3 (37.5%)	1.00
No	10 (66.7%)	5 (62.5%)	
Hypertension			
Yes	12 (80.0%)	8 (100.0%)	0.53
No	3 (20.0%)	0 (0.0%)	
CKD Stage (MDRD)			
Stage III	5 (31.3%)	5 (62.5%)	0.20
Stage IV/V‡	11 (68.8%)	3 (37.5%)	

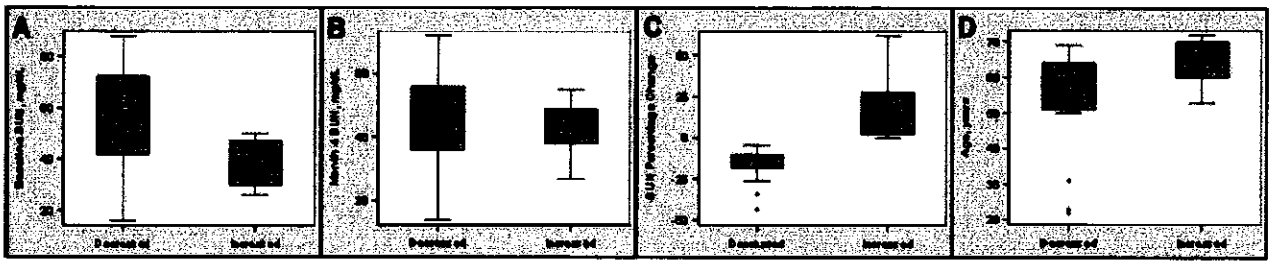
RESULTS

Twenty-seven plasma samples were selected for metabolomics analysis from a previously reported dose escalation study of

TABLE 3. Clinical Data. Values reported are the median (IQR). *Two missing values for CRP at month 4 and one missing value for Potassium at baseline. **Wilcoxon Rank Sum Test. †% Change calculation: (Month 4 – Baseline)/Baseline * 100.

Clinical Measurement*	Decreased BUN (n=16)	Increased BUN (n=8)	p-value**
BUN, mg/dL			
Baseline	58.5 (31.0)	36.5 (17.0)	**0.03**
Month 4	48.5 (20.0)	43.5 (11.0)	0.49
% Change†	-12.5 (7.8)	3.3 (25.7)	**0.0007**
Systolic Blood Pressure, mmHg			
Baseline	130.0 (14.0)	129.0 (23.0)	0.90
Month 4	135.0 (30.0)	125.0 (23.0)	0.26
% Change†	2.19 (11.9)	-4.1 (17.2)	0.13
Diastolic Blood Pressure, mmHg			
Baseline	69.0 (17.0)	70.0 (9.0)	0.41
Month 4	73.0 (12.0)	70.0 (13.0)	0.54
% Change†	7.4 (22.5)	-4.5 (18.1)	0.15
Creatinine, mg/dL			
Baseline	2.4 (1.2)	2.0 (0.8)	0.10
Month 4	2.4 (1.4)	2.0 (0.8)	0.43
% Change†	-1.9 (13.8)	-1.7 (26.9)	0.29
CRP, mg/L			
Baseline	0.3 (0.4)	0.3 (0.4)	0.68
Month 4	0.3 (0.5)	0.3 (0.3)	0.64
% Change†	0.0 (45.0)	0.0 (25.0)	0.61
Hemoglobin, mg/dL			
Baseline	10.4 (2.10)	11.2 (1.65)	0.35
Month 4	10.7 (2.25)	11.0 (0.90)	0.37
% Change†	2.5 (11.60)	-0.5 (5.83)	0.74
Potassium, mmol/L			
Baseline	4.7 (1.00)	4.6 (1.10)	0.72
Month 4	4.4 (0.60)	4.6 (0.50)	0.61
% Change†	-4.5 (10.40)	2.2 (11.26)	0.34

FIGURE 1. Distribution of BUN and Age by Phenotypic Group. A. Boxplots showing the distribution of BUN at baseline. B. Boxplots showing the distribution of BUN at 4 Months. C. Boxplots showing the distribution of percent change of BUN. D. Boxplots showing the distribution of age.



the probiotic Renadyl™ (Ranganathan et al., 2013). Of these three were excluded and the remainder were stratified by change in BUN over four months of supplementation. Sixteen subjects had a decrease in BUN at the end of the 4-month trial, and BUN for the remaining 8 subjects was at or above the baseline level. Figure 1 shows the distribution of BUN at baseline, month 4, and the percentage of change as well as the distribution of age. The study population is described in Table 2. Age was statistically different between the two groups (Figure 1, p=0.045). There were no statistical differences for

TABLE 4. NMR Bins that differentiate decreased and increased BUN groups. *Jackknifed confidence interval does not include 0, **Wilcoxon Rank Sum Test, †Positive fold change means the bin peak intensity of increased BUN is higher than decreased BUN.

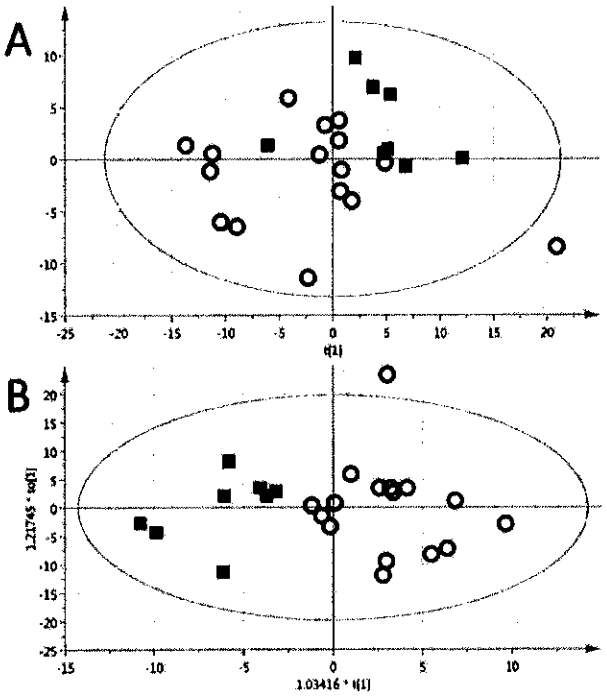
Associated Metabolites	Bin [ppm range]	VIP*	p-value**	Fold Change†
Lipoproteins, Lactate, Threonine	[1.28 .. 1.34]	5.4	0.018	1.1
O-Acetylcholine, O-Phosphocholine	[3.20 .. 3.26]	4.9	0.013	-1.2
Creatine, Creatinine	[2.99 .. 3.05]	4.2	0.006	-1.4
Glucose	[5.18 .. 5.24]	2.8	0.009	1.2
Choline, O-Acetylcholine	[3.17 .. 3.20]	2.8	0.018	-1.2
Creatinine	[4.02 .. 4.07]	2.4	0.030	-1.5
Betaine, Creatine	[3.91 .. 3.96]	2.3	0.092	-1.2
Malonate	[3.08 .. 3.11]	1.9	0.257	-1.4
Proline	[2.04 .. 2.08]	1.8	0.030	-1.1
1,3-Dimethylurate	[3.28 .. 3.31]	1.7	0.209	-1.4
Ethylene glycol	[3.65 .. 3.67]	1.3	0.071	-1.3
Trimethylamine, Dimethylglycine	[2.88 .. 2.93]	1.2	0.025	-1.5
Unknown	[3.60 .. 3.62]	1.2	0.035	-1.5
Tartrate	[4.28 .. 4.34]	0.9	0.025	-2.3

TABLE 5. Metabolites and related pathways that differentiate the BUN study groups.

Metabolite	Pathway information	Microbial Related	Related to Renal Function
1,3-Dimethylurate	Theophylline Metabolism	--	--
Betaine	Choline Metabolism	(Nicholson, Holmes et al. 2012)	(Grunewald and Eckstein 1995)
Choline	Choline Metabolism	(Nicholson, Holmes et al. 2012)	(Rhee, Clish et al. 2013)
Creatine	Creatine and Creatinine Metabolism	(Wrong 1978)	In affected pathway
Creatinine	Creatine and Creatinine Metabolism	(Wrong 1978)	(Levey, Perrone et al. 1988)
Dimethylglycine	Choline Metabolism	(Nicholson, Holmes et al. 2012)	(McGregor, Dellow et al. 2001, Vanholder, De Smet et al. 2003)
Ethylene glycol	Exogenous	--	--
Glucose	Carbohydrate Metabolism	(Zheng, Xie et al. 2011)	(de Boer 2008)
Lactate	Carbohydrate Metabolism	(Zheng, Xie et al. 2011)	--
Lipoproteins	Nutrient Absorption and Energy Regulation	(Krajmalnik-Brown, Ilhan et al. 2012)	(Lin, Khetarpal et al. 2015)
Malonate	Pyrimidine metabolism	(Krajmalnik-Brown, Ilhan et al. 2012)	(Rhee, Souza et al. 2010)
O-Acetylcholine	Choline Metabolism	(Nicholson, Holmes et al. 2012)	In affected pathway
O-Phosphocholine	Choline Metabolism	(Nicholson, Holmes et al. 2012)	In affected pathway
Proline	Arginine and proline metabolism	(Zheng, Xie et al. 2011)	(Tizianello, De Ferrari et al. 1980)
Tartrate	Exogenous	--	--
Threonine	Essential amino acid	(Puiman, Stoll et al. 2013)	(Tizianello, De Ferrari et al. 1980, Rhee, Clish et al. 2013)
Trimethylamine	Choline Metabolism	(Nicholson, Holmes et al. 2012)	(Duranton, Cohen et al. 2012)

BMI, gender, race/ethnicity, diagnosis of diabetes, diagnosis of hypertension, and CKD stage. The clinical data at baseline, month 4, and the percentage change during the trial is described Table 3. There was a statistical difference for BUN (Figure 1) at baseline (p=0.03) with median BUN of 58.5 mg/dL (IQR=31.0) for the decreased BUN group and 36.5 mg/dL (IQR=17.0) for the increased BUN group. At the 4-month visit the median BUN had decreased to 48.5 mg/dL (IQR=20.0) for the decreased BUN group and increased to 43.5 mg/dL (IQR=11.0) for the increased BUN group so that there was no longer a statistical difference between the two groups (p=0.49). There was a significant difference in the percentage

FIGURE 2. The PCA (A) of the plasma samples differentiated subjects with increased BUN (black squares, upper right quandrant) or decreased BUN (empty circles). The OPLS-DA (B) had a 100% correct classification and Fisher's probability of 1.4 E-06. R2X(cum) = 0.40, Q2(cum) = 0.31



change of BUN (p=0.0007) with the decreased BUN group having a median change -12.5% (IQR=7.8) and the increased BUN group having a median change of 3.3% (IQR=25.7). There were no statistical differences for systolic blood pressure, diastolic blood pressure, creatinine, CRP, hemoglobin, and potassium at measured at baseline and month 4 as well as no statistical differences for the percent change in measurements between month 4 and baseline.

Metabolomics using BUN as phenotypic anchor

Unsupervised multivariate analysis (PCA) of plasma samples differentiated subjects with increased BUN (Figure 2a, black squares, upper right quandrant) or decreased BUN (empty circles). A supervised analysis (OPLS-DA) resulted in 100% correct classification of BUN (Figure 2b), with a Fisher's probability of 1.4 E-06. The metabolites that differentiated the BUN groups are listed in Table 4. Many of the metabolites that comprise the marker profile are significantly different ($p<0,05$) between the BUN groups. These metabolites are related to pathways of carbohydrate metabolism, and energy metabolism and regulation, and choline metabolism (Table 5). Additionally, a supervised multivariate analysis (OPLS-DA) was able to differentiate the Stage III profiles from Stage IV/V, as shown in Figure 3. The differentiating metabolites for the progression of disease are listed in Table 6, and Table 7 highlights the metabolites that are unique to the differentiation of the BUN groups and of disease progression.

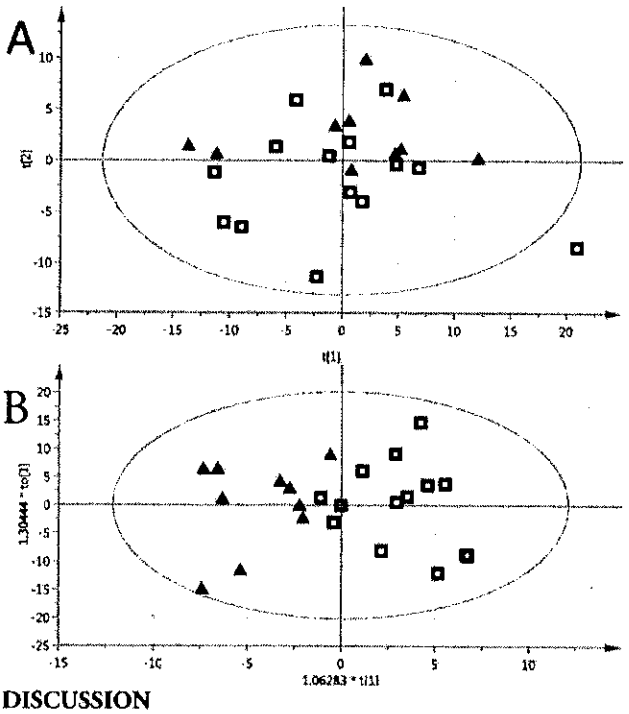
TABLE 7. Metabolites that are unique to differentiating the BUN groups and CKD stage.

Metabolites Unique to ΔBUN Phenotype	Metabolites Unique to CKD Stage Phenotype
Betaine	Alanine
Dimethylglycine	Glycerol
Ethylene glycol	Lipids
Lactate	N-Acetylamino acids
Lipoproteins	Propylene glycol
Malonate	Unknown
O-Phosphocholine	
Tartrate	
Threonine	
Trimethylamine	

TABLE 6. NMR Bins that differentiate CKD stages III and IV/V[1]. *Jackknifed confidence interval does not include 0, **Wilcoxon Rank Sum Test, †Positive fold change means the bin peak intensity of stage III is higher than stage IV/V. [1]One subject was determined to be Stage V after meeting inclusion criteria and remained pre-ESRD.

Associated Metabolites	Bin [ppm range]	VIP*	p-value**	Fold Change†
Glucose, 1,3-Dimethylurate	[3.43 .. 3.48]	3.9	0.71	-1.0
Unknown	[0.82 .. 0.85]	3.2	0.01	1.3
Alanine	[1.42 .. 1.48]	3.2	0.06	1.1
Proline, N-Acetylamino acids	[1.98 .. 2.04]	3.1	0.39	1.1
Glucose	(6 bins)	1.2 – 3.0	0.07 - 0.91	(-1.1) – (1.0)
Creatine, Creatinine	[2.99 .. 3.05]	2.7	0.14	-1.2
Propylene glycol	[1.09 .. 1.14]	2.7	0.04	1.5
Choline, O-Acetylcholine	[3.17 .. 3.20]	1.8	0.10	-1.2
Glycerol	[3.62 .. 3.65]	1.4	0.28	-1.1
Lipids	[1.14 .. 1.17]	1.0	0.12	1.2

FIGURE 3. The PCA (A) and supervised multivariate analysis (OPLS-DA) (B) of the Stage 3A/B (black triangles) and Stage 4/5 (empty squares) profiles. CKD stage assignment is based on



DISCUSSION

These results indicate a subset of patients with CKD III or IV respond positively to probiotic use as measured by the primary outcome of a decrease in BUN after 4 months. A metabolic phenotype was present prior to starting the regimen that has potential to predict response. This phenotype has characteristics unique to the BUN response phenotype when compared to kidney function.

BUN was chosen as the primary outcome measure because it was the only clinical measure to change significantly across all study samples over the course of probiotic use. This observation was consistent with a previous pilot that found BUN was the measure of kidney function most affected by probiotics (Ranganathan et al., 2009). The observed change in BUN is an important outcome of the clinical trial (Ranganathan et al., 2013), because BUN is a marker of kidney function and an indicator of protein carbamylation, an independent mortality risk factor among kidney failure patients (Berg et al., 2013). The observed decrease from 58.5 mg/dL to 48.5 mg/dL BUN among responders can be considered clinically encouraging; however, the four month time scale is too short to evaluate the impacts on clinical decisions such as starting dialysis. The BUN concentration decreased for 16 of the study participants and increased for 8. Another clinical trial has probed the use of probiotics and observed differences in the gut microbiome response in their subject population (Wang et al., 2014).

The first observed characteristic of the predictive metabolic phenotype was a higher BUN concentration at baseline in the decreased BUN group. Many probiotics, including the probiotic used in this study, include urease producing bacteria in an effort to increase degradation of urea (Vaziri et al., 2015). Presumably, bacterial ureases are driving the observed change in BUN. A recent review questioned the safety of this approach (Vaziri et al., 2015) and hypothesized that the conversion of urea to ammonium hydroxide could raise the pH of the intestinal lumen and damage the intestinal epithelial barrier (Vaziri et al., 2013). While not indicative of local pH changes, a previous pilot study (Ranganathan et al., 2009), however, demonstrated lower fecal pH with probiotic use. This can be explained by consumption of ammonia for biosynthesis and simultaneous production of lactic acid by *L. acidophilous* which is also known to lower circulating simple aliphatic amines (Dunn et al., 1998).

Metabolic profiling revealed a phenotype in the baseline samples that was predictive of the subset of study participants that would respond to probiotic supplement use (decreased BUN group). The large majority of metabolites contributing to this phenotype (Table 5) have been described in the gut microflora and kidney disease literature, which include metabolites involved in creatinine, choline, carbohydrate, and amino acid pathways. Creatinine values are generally expected to correlate with BUN when changes are due to glomerular filtration rate. Creatinine is a waste product of muscle metabolism which uses the creatine-phosphocreatine buffer as an energy source, and may build up in the blood if glomerular injury has occurred. For this reason, serum creatinine concentrations and creatinine glomerular filtration rate (GFR) are commonly used as clinical indicators of kidney function (Hosten, 1990). The metabolomics analysis found significantly lower integrals in both creatinine NMR bins ($p=0.006$ and $p=0.03$) for the increased compared to decreased BUN group, consistent with the clinical measurement ($p=0.10$, Table 3). Creatinine is degraded by gut microflora (Levey et al., 1988) and has been found slightly elevated in plasma concentrations in conventional compared to germ-free mice (Wikoff et al., 2009), while both creatine and creatinine were found to be higher in feces of rats treated with antibiotics (Zheng et al., 2011).

Choline metabolism is important for osmoregulation within the kidney. The kidney synthesizes osmolytes betaine (Grunewald and Eckstein, 1995) and glycerophosphocholine (Zablocki et al., 1991) from choline to retain water and respond to changes in tonicity. Choline deficiency is associated with damage to the kidney (Denninghoff et al., 2014) and changes in gut microbial ecology (Spencer et al., 2011). Due to its metabolism in proximal tubular cells, others have hypothesized that elevated plasma choline may signal tubulointerstitial dysfunction, a pathology which is even more highly correlated with CKD prognosis than

glomerular injury (Rhee et al., 2013). Trimethylamine and dimethylglycine are known uremic solutes (McGregor et al., 2001;Duranton et al., 2012), and metabolism of choline by the gut microbiome is a major contributor to their synthesis (al-Waiz et al., 1992;Matsumoto et al., 2012) The presence of dimethylglycine in the intestinal lumen has also been positively correlated with the presence of *Enterobacteriaceae* and negatively with *Lactobacillus* sp. (Matsumoto et al., 2012). There are several genetic polymorphisms that are correlated with choline deficiency (Zeisel, 2006), such as a 5,10-methylenetetrahydrofolate dehydrogenase-1958A allele. Carriers were more likely to develop a choline deficiency than noncarriers in a healthy population (Kohlmeier et al., 2005). Additional insight would come from understanding the influence of this allele on probiotic response in a validation study population. Choline monitoring in CKD patients has not been attempted previously; however, this would be straightforward and modifiable through dietary intervention.

Carbohydrate metabolism deficiencies were observed in uremia (Perkoff et al., 1958) and chronic renal failure (Hampers et al., 1966) as far back as 1958, but it is not clear if this glucose imbalance is a part of the pathology, progression of disease, or both (de Boer, 2008). The genus *Bifidobacterium* has been studied in detail because the constituent genomes encode a large number of carbohydrate-modifying enzymes (Pokusaeva et al., 2011). *S. thermophilius* is a significant producer of lactate in yogurt cultures (Gezginc et al., 2015), while *L. acidophilus* is noted for its carbohydrate metabolism (Goh and Klaenhammer, 2014) and lactate consumption (Sulek et al., 2012).

The renal arteriovenous metabolite gradients of amino acids indicate that the kidney actively absorbs and metabolizes proline from arterial blood and releases the essential amino acid threonine (Tizianello et al., 1980). An 80-90% reduction in both activities was observed in patients with reduced kidney function. Proline has been observed to increase in the urine of rats after treatment with antibiotics (Zheng et al., 2011) and threonine levels were higher in serum after antibiotic treatment of neonatal pigs (Pulman et al., 2013). The majority of threonine is used to support intestinal mucin synthesis and is critical to maintaining intestinal barrier function (van der Schoor et al., 2007).

The higher BUN and trend to higher creatinine levels measured in the decreased group raises the possibility that a probiotic supplement may be most effective for CKD III-IV patients with poorer kidney function. However, when the CKD stage was used to determine the metabolic phenotype related to kidney function (Table 6), the metabolites most important for separating Stage III from Stage IV/V subjects had some overlap, but were not the same as those most important for the change in BUN (Table 7). Of the metabolites unique to predicting BUN response, choline metabolism represents the majority of the distinguishing bins.

While metabolomics studies have investigated metabolic signatures of uremia (Duranton et al., 2012) or disease progression (Rhee et al., 2013), not many studies have reported changes in metabolic profiles with progression from CKD stage III to IV (Krajmalnik-Brown et al., 2012;Lin et al., 2015). Of those metabolites observed to be unique to the CKD stage model, alanine (Rhee et al., 2013), lipids (Rhee et al., 2010), and N-acetylamino acids (Sekula et al., 2015) have been reported in previous CKD metabolomics studies. Notably, other markers of renal dysfunction (Niewczas et al., 2014) including dimethylarginine, arginine, citrulline, ornithine, spermidine, tryptophan, kynurenine, serotonin, indoxyl-sulfate, tyrosine, phenylalanine, p-cresol, TMAO and acylcarnitines were not determined to be important for differentiating CKD stage in this data.

The predictive metabolic phenotype may reflect the status of the gut microbiome at baseline, and the lack of change in BUN in the increased BUN group may be a reflection of a more resilient gut microbiome less altered by poor kidney function. Mechanistic effects of this dietary probiotic supplement use remain to be worked out in detail.

Variables that could influence the generation of gut originated metabolites, diet and medications were kept constant for the duration of the study. However, no food intake diaries were kept, and CKD patients are often provided with nutritional recommendations such as restricting intake of protein, sodium and potassium. Any dietary changes were expected to be random, but an independent study could verify this.

The probiotic supplementation may cause changes in the diversity and/or function of the gut microbiome, leading to changes in BUN, metabolite profiles, kidney function, and clinical outcomes. A future study measuring each in parallel could lead to further mechanistic insights. The predictive metabolic phenotype warrants validation in a larger cohort and longer study, particularly to understand the generalizability of the phenotype to other prebiotic and probiotic treatments. The current study also demonstrates the feasibility of a larger study with serial microbiome measurements for a mechanistic understanding of probiotic treatment related improvements in kidney function and personalized intervention strategies in CKD.

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