ORIGINAL RESEARCH

The Impact of Synbiotic Treatment on the Levels of Gut-Derived Uremic Toxins, Inflammation, and Gut Microbiome of Chronic Kidney Disease Patients—A Randomized Trial

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Objective: Altering dysbiotic gut flora through synbiotic supplementation has recently been recognized as a potential treatment strategy to reduce the levels of gut-derived uremic toxins and decrease inflammation. Assessing its efficacy and safety has been the main goal of our randomized, double-blind, placebo-controlled study.

Methods: A total of 34 nondialyzed chronic kidney disease patients, aged ≥18 years, with an estimated glomerular filtration rate between 15 and 45 mL/minute, were randomized either to an intervention group (n = 17), receiving synbiotic (*Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium lactis*, 32 billion colony forming units per day plus 3.2 g of inulin), or control group (n = 17), receiving placebo during 12 weeks. The impact of treatment on the dynamic of serum levels of gut-derived uremic toxins, total serum indoxyl sulfate, p-cresyl sulfate, and trimethylamine N-oxide, was defined as the primary outcome of the study. Secondary outcomes included changes in the stool microbiome, serum interleukin-6 levels, high-sensitivity C-reactive protein, estimated glomerular filtration rate, albuminuria, diet, gastrointestinal symptom dynamics, and safety. Serum levels of uremic toxins were determined using ultraperformance liquid chromatography. The stool microbiome analysis was performed using the 16S ribosomal ribonucleic acid gene sequencing approach.

Results: Synbiotic treatment significantly modified gut microbiome with *Bifidobacteria*, *Lactobacillus*, and *Subdoligranulum* genera enrichment and consequently reduced serum level of indoxyl sulfate (ΔIS –21.5% vs. 5.3%, $P < .001$), improved estimated glomerular filtration rate (ΔeGFR 12% vs. 8%, $P = .029$), and decreased level of high-sensitivity C-reactive protein (–39.5 vs. –8.5%, $P < .001$) in treated patients. Two patients of the intervention arm complained of increased flatulence. No other safety issues were noted.

Conclusion: Synbiotics could be available, safe, and an effective therapeutic strategy we could use in daily practice in order to decrease levels of uremic toxins and microinflammation in chronic kidney disease patients.

Keywords: Chronic Kidney Disease; Cardiovascular Burden; Gut-derived Uremic Toxins; Synbiotic; Microbiome; Inflammation

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Introduction

THE GUT MICROBIOME consists of more than 100 trillion bacteria and plays an important role in normal body functioning, especially in immunity and metabolic homeostasis. There is increasing evidence that gut microbiome alteration can affect multiple organ systems and also lead to numerous chronic diseases, such as chronic kidney disease (CKD).[1] The connection between the kidneys and gut microbiome, the gut-kidney axis, is bidirectional. On the one hand, accumulation of urea and volume overload affect the composition of gut microbiome and increase permeability of the gut epithelial barrier, which in return boosts the production of gut-derived uremic substances with significant renal and vascular toxic effects.[2] The buildup of approximately 90 different substances has been recognized as a result of reduced kidney function. Most of those toxins are gut derived through the process of proteolytic fermentation by an altered gut microbiome.[3] The most studied ones are indoxyl sulfate (IS), p-cresyl sulfate (pCS), and trimethylamine N-oxide (TMAO).[4]

CKD presents a major and steadily growing health problem worldwide. CKD mostly manifests itself as a silent disease paralleled by irreversible changes in vascular structures, so up to half of all CKD patients are reported to die from cardiovascular (CV) complications.[5] Therefore, prevention of CV events is among the main goals in CKD management. Lately, the focus of investigators has been on potentially modifiable, "nontraditional," CV risk factors in CKD patients. One of the promising candidates is altering dysbiotic gut flora through synbiotic supplementation in order to reduce the levels of gut-derived uremic toxins and decrease chronic microinflammation.[6]

Up to date, a total of 10 randomized, double-blind, placebo controlled studies have evaluated the efficacy and safety of prebiotics and probiotics on the level of uremic toxins and inflammatory status (Table S1).[7-16] A published meta-analysis on this subject declared that owing to the small number of published studies, it is difficult to conclude if this kind of intervention has an important effect on the level of uremic toxicity and inflammation.[17]

Therefore, in order to increase the pool of knowledge on the subject, we performed our single-center, randomized, double-blind, placebo-controlled study with the aim of assessing the efficacy and safety of synbiotic treatment in reducing the levels of gut-derived uremic toxins and serum inflammatory markers and its impact on gut microbiome, with controlled factors such as diet and antibiotic usage.

Materials and Methods

Study Design and Participants

Our single-center study was designed as a double-blind, placebo-controlled randomized trial that included nondialyzed CKD patients, aged ≥18 years, with estimated glomerular filtration rate (eGFR) between 15 and 45 mL/minute. The exclusion criteria for the study were participation in another trial, inability to communicate, previous renal transplantation, inflammatory bowel disease, previous intestinal resection, radiotherapy, or course of antibiotic, probiotic, and immunosuppressive therapy 2 weeks prior to and during the trial. All patients that progressed to dialysis during the study period were also excluded from the per-protocol population. Patients were asked to sign informed consent before enrollment and participation in the trial. The institutional ethics comitee approved the protocol of our trial. The study was conducted according to the guidelines of the Declaration of Helsinki of 1975.

The sample size of the intention-to-treat population (n = 44) was calculated using a Raosoft software program and based on the count of the population of predialysis CKD outpatients regularly followed up (n = 300), the power of 90% to detect a 30% change in the level of uremic toxins, with an error level of 0.05 and predicted dropout rate of about 30%.[18]

Diet

Following the signing of an informed consent, patients underwent counseling with a qualified dietitian concerning their diet during the study period. The diet was based on Kidney Disease Quality Initiative recommendations.[19] The diet plan included 2 weeks of a run-in period and a 12-week treatment period. Each patient was given a cookbook containing 150 Mediterranean diet-based recipes with nutritive information and options for each meal. Patients were advised to have 4-5 daily meals. A 7-day meal plan example was issued to each patient at the beginning of each week of the trial. Patients were instructed how to calculate daily calories, carbohydrate, protein, phosphorus, sodium, and potassium intake and advised to keep the daily intake of calories between 25 and 30 kCal/kg/day, protein intake between 0.5 and 0.7 g/kg/day and under 10% of daily calorie intake, phosphorus intake approximately 500 mg a day, calcium intake arround 800 mg/d, sodium intake of less than 2.3 g/d, and potassium intake of less than 4 g/d. Electrolite intake was individually prescribed based on blood analysis performed after a run-in period. Patients were asked to keep a diet diary, which was used to assess diet adherence. FoodWorks 10 software by Xyris Software (Australia) was used to analyze dietary data.

Randomization

After a 2-week run-in period under prescribed diet, patients were randomized into 2 groups, one receiving synbiotic therapy, and the other receiving placebo. Randomization was performed by an external statistical consultant using computer-based randomization by the blocks of 4 provided by Sealed Envelope Ltd 2020.[20] After the randomization, the research personnel were informed about the therapy kit number allocated for each patient. The randomization data were maintained on a secure

server, not available to patients and personnel performing the trial until all results came in. Kit packaging and numeration were performed offsite.

Intervention

The synbiotic arm of patients underwent daily treatment that consisted of 2 pills, each containing 16 billion colonies of *Lactobacillus acidophilus CBT LA1* (4×10^9), *Lactobacillus casei CBT LC5* (4×10^9), and *Bifidobacterium lactis CBT BL3* (8×10^9) and 1.6 g of inulin, that were meant to be taken once daily before breakfast. Probiotic selection was based on previous trials of similar design which mostly used mixed cultures of *Bifidobacterium* and *Lactobacillus spp.*[7-16] *Bifidobacterium lactis*, one of the most documented probiotic cultures, was chosen due to its gastric acid and bile tolerance, high level of adhesion, and pathogen inhibition ability, even more pronounced when used in combination with *Lactobacillus acidophilus*.[21] *Lactobacillus casei* was chosen for its negative effects on pCS level, described in previous studies.[7,8] The dosage of synbiotic supplement was based on previous findings of good efficacy and stool recovery of *Bifidobacterium lactis* without described adverse effects if used at a dosage of 10^{10} or higher.[22]

The placebo arm of patients received the same number of identical pills containing maltodextrin powder and received the same regimen instructions. The duration of treatment period was 12 weeks. Kits containing 56 pills were issued to patients on visits that were scheduled every 4 weeks. Patients were asked to bring along all remaining capsules and their diet diaries to center, for each visit. Nonadherence to study protocol was defined as more than one-tenth of all issued capsules not taken by participant, and those patients were excluded from the per-protocol population.

Outcome Measures

The impact of treatment on the dynamic of serum levels of gut-derived uremic toxins, total serum IS, pCS, and TMAO, was defined as the primary outcome of the study. Secondary outcomes included changes in the stool microbiome, serum interleukin-6 (IL-6) levels, high-sensitivity C-reactive protein (hsCRP), eGFR, albuminuria, diet, gastrointestinal symptom dynamics, and safety.

The stool, blood, and urine samples of all patients were obtained after 2 weeks of run-in and after the 12-week treatment. The levels of hsCRP and IL-6 were analyzed by enzyme-linked immunoadsorbent assay. Renal function and progression of renal failure were determined by using online The Modification of Diet in Renal Disease formula.[23] Albuminuria was analyzed from spot urin sample. Patients' gastrointestinal symptom dynamic was monitored using the Gastrointestinal Symptom Rating Scale (GSRS) questionnaire, concerning presence and severity of 5 dimensions of gastrointestinal symptoms: abdominal pain, reflux, indigestion, constipation, and diarrhea.[24] All patients were interviewed at the beginning and after the end of the treatment period. We also measured body mass index (BMI) for each patient before and after the treatment period.

Determination of Uremic Toxins

The Waters ACQUITY ultraperformance liquid chromatography system (Waters Corporation, Milford, MA) coupled with an FLD detector 2475 was used for the quantitative determination of total IS and pCS in patient serum samples before and after treatment. Acquisition of data was performed using Waters Empower 2 software (Milford, MA). The extraction of total IS and pCS from serum samples was performed after protein precipitation with ethanol, using 100 μL of samples and 300 μL of ethanol (100%) according to the procedure described by Pretorius et al.[25] Extracted samples were filtered using 0.22-μM nylon filters (Phenomenex, Torrance, CA) and then separated on an ACQUITY UPLCTM BEH C18 column (1.7 μm, 100 mm $\times$ 2.1 mm) using linear gradient elution, with mobile phase A consisting of 50-mM ammonium formate (pH 5) and mobile phase B consisting of 100% acetonitrile. Gradient started with 95% A for 1.5 minutes, then from 75% to 30% A in the next 1 minute, then from 30% to 1% A for 0.4 minute, and was held at 1% A for 2.9 to 3.6 minutes. At 3.8 to 7 minutes, A gradually increased from 15% to 95%. The flow rate was 0.25 mL/minute, column T 45°C, and injection volume 5 μL. Fluorescence detection was monitored at specific excitation/emission wavelengths λex = 300 nm and λem = 390 nm.[25] Standard solutions of IS and pCS were prepared by serial dilution of the stock solution.

Ultrahigh performance liquid chromatography-tandem mass spectrometry using an Acquity ultraperformance liquid chromatography coupled to a MicroMassQuattro Premier XE mass spectrometer (Waters Corporation, Milford, MA) was performed in order to determine the total serum levels of TMAO, according to the procedure described by Awwad et al.[26] The samples were separated using a gradient mobile phase with a mixture of 15-mmol/L ammonium formate (pH 3.5) as solvent A and acetonitrile as solvent B. Gradient conditions were 0.0–2.0 minutes, 10–30% A; 2.0–3.5 minutes, 30% A; 3.6–4.6 minutes, 40% A; and 4.7–6.0 minutes, 10% A. We used the following settings: flow rate, 0.4 mL/minute; sample injection volume, 3 μL (partial loop mode); column temperature, 30°C; sample temperature, 4°C; total runtime, 6 minutes.

Stool Microbiome Determination

Patients were provided with stool containers and asked to bring fresh stool samples (≤6 hours) to the center. Stool samples were stored at −80°C prior to microbiome profile analysis. Total genome DNA from frozen fecal samples was performed with the ZR Fecal DNA MiniPrep Kit (Zymo Research Corp., Irvine, CA), according to the manufacturer's instruction. Isolated DNA from all samples was stored at −20°C after PicoGreen DNA concentration measurements on Qubit fluorometer (ThermoFisher/Invitrogen, Waltham, MA). All samples were diluted to a

concentration of 5 ng/μL in 10-μL final volume and used for 16s rRNA amplicon sequencing, targeting the V3-V4 hypervariable region. Paired-end sequencing was performed on NovaSeq 6000 PE250-Illumina platform by Novogene Co., Ltd (Hong Kong, China). The libraries were sequenced on an Illumina MiSeq platform (Illumina, San Diego, CA) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The raw reads were demultiplexed, quality-filtered by Usadellab Trimmomatic (Germany), and analyzed using the Quantitative Insights Into Microbial Ecology pipeline (qiime.sourceforge.net) as described by Caporaso et al.[27] Operational taxonomic units (OTU) clustering and species annotation were performed. Sequence analysis was performed by using Uparse software (Uparse v7.0.1001, Tiburon, CA).[28] Sequences with ≥97% similarity were assigned to the same OTUs. The representative sequence for each OTU was screened for annotation. For each representative sequence, mothur software was used against the small subunit ribosomal ribonucleic acid database of SILVA Database for species annotation at each taxonomic level (Threshold: ∼0.8 to 1) (kingdom, phylum, class, order, family, genus, species).[29,30] OTU abundance information was normalized using a standard of sequence number corresponding to the sample with the fewest sequences. The Shannon and Simpson indexes were calculated in R as a measure of alpha diversity. Beta diversity was assessed using weighted unifrac as a distance measurement, plugin with p-method "anosim," following 999 permutations for determining the differences between the groups. Differences in β-diversity between the groups were visualized using principal coordinates analysis biplots. The diversity boxplot was created using the plot function in the scikit-bio package using Python 3.9.

Core method from package "stats" in R and ggplot2 were used for Spearman's rank correlation matrix generation and visualization.

Safety Data

All adverse events were documented and reported to the institutional ethics committee, which initially approved the protocol of our study. Serious adverse events were defined as any undesirable sign, symptom, or medical condition that was fatal or life-threatening, required hospitalization, or resulted in significant patient disability or death.

Statistical Analysis

The statistical analyses were performed using appropriate tests with SPSS 23.0 (IBM Corp., Armonk, NY) and R Core Team Software (2020). All data were presented as mean ± standard deviation if normally distributed; otherwise, it was presented as median (25%-75%). To detect differences in basic characteristics and the dietary intakes between the 2 groups of patients, we applied Pearson c2 and Fisher exact test for categorical variables and 2-tailed independent samples Student's t-tests for normally distributed numerical variables. All intergroup comparisons were performed using either Student's t-test for normally distributed data or the Mann-Whitney U test for not normally distributed data. *P* values under 0.05 were considered statistically significant. Graphs are made using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA).

Results

Our single-center trial was performed during the period from October 1, 2019, to February 1, 2020. After the initial assessment of 106 patients, 44 patients who fulfilled inclusion and exclusion criteria were recruited for the study (Figure 1). Patients were randomized to the intervention arm (n = 22) receiving synbiotic treatment and the control arm (n = 22) receiving placebo. One patient from the control arm was lost to follow-up after moving abroad. Two patients from the control arm started hemodialysis due to uncontrolled hypervolemia. Four patients, 2 from each arm, took less than 80% of all issued pills, and therefore were not included to the per-protocol population. Two patients from the intervention arm took antibiotics during the study for respiratory tract infections and were also excluded from the per-protocol population. One patient from the intervention arm did not deliver an end-point fresh stool sample and was not included in the per-protocol population.

Basic characteristics of both groups of patients are represented in Table 1. No major differences in terms of age, gender, comorbidities (diabetes mellitus, hypertensive nephrosclerosis), BMI, previous medication usage, eGFR, blood urea nitrogen, and serum potassium have been observed. Similar conclusions can be made based on the intention-to-treat population (Table S2).

Primary Outcomes

In the intervention arm, 12 weeks of synbiotic therapy significantly reduced total serum IS (73.1 mol/L vs. 55.3 mol/L, $P = .001$) and pCS (136.7 mol/L vs. 128 mol/L, $P = .0153$) levels (Figure S1). If we compare uremic toxin dynamics between the intervention and placebo-controlled arms of patients, the effect of synbiotic therapy was of significantly greater magnitude (ΔIS −21.5% vs. 5.33%, $P < .001$). If we compare 2 groups of patients, the synbiotic therapy impact on pCS and TMAO level change was not statistically different in comparison to placebo (Table 2). If we take into account the whole intention-to-treat population, we would observe a statistical difference not only in IS dynamic (ΔIS −14.6% vs. 5.3%, $P = .002$) but also in TMAO (ΔTMAO −5.3% vs. 1.6%, $P = .039$) (Table S3).

Secondary Outcomes

Biochemical Parameters

Both groups of patients had baseline hsCRP levels above the normal range. Although patients in both arms lowered their levels of hsCRP, the level of reduction was significantly higher in the intervention arm than that in placebo

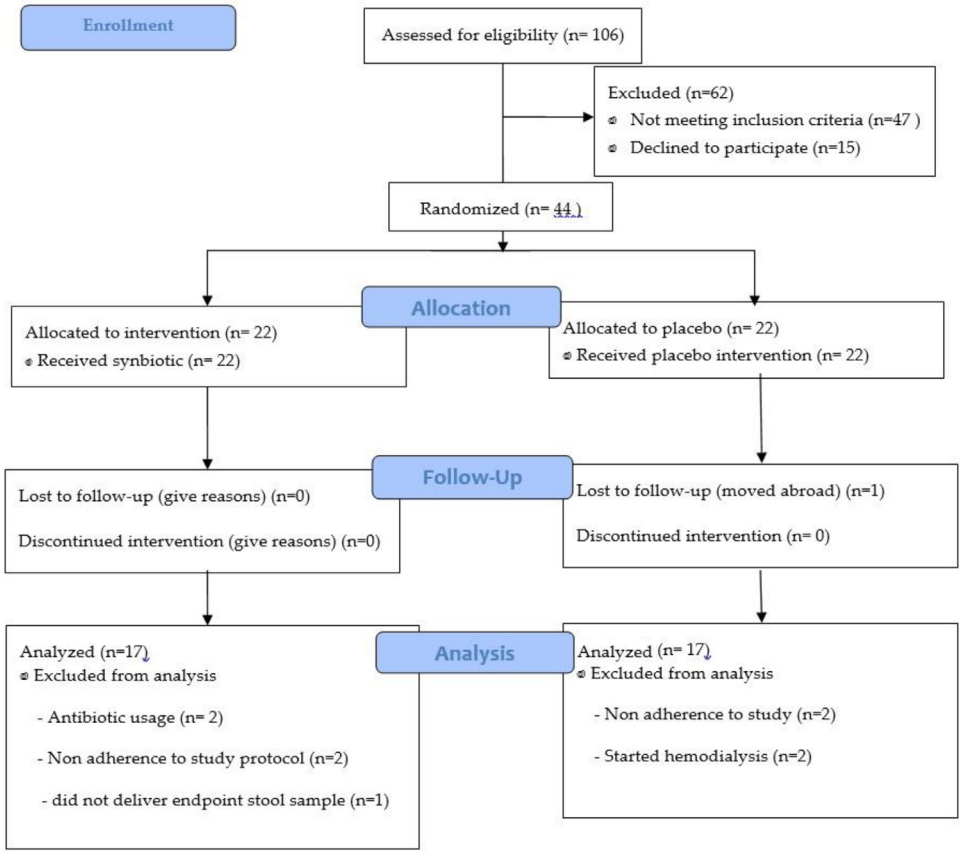

Figure 1. Patient flowchart.

(ΔhsCRP% −39.5 vs. −8.5, $P < .001$). The eGFR dynamic, as one of the markers of kidney function, showed improvement in both groups of patients, with a more pronounced effect of synbiotic therapy than placebo (ΔeGFR% 12.5 vs. 8, $P = .029$). In terms of serum potassium, blood urea nitrogen, IL-6 levels, and albuminuria, no significant advantage of synbiotic therapy was noted (Table 3). The effect of synbiotics on hsCRP levels can also be observed but to a lesser extent in the intention-to-treat population, but eGFR dynamic fell out of statistical significance (Table S4).

Stool Microbiome Dynamic

All stool samples delivered from the per-protocol population, both at baseline and endpoint, were adequate. The diversity coverage of the microbiome was good—a total of 12,515,472 reads—mean 184,051 reads per sample, with goods coverage values over 95%. The mean Shannon and Simpson index coefficients significantly changed between baseline and endpoint in the synbiotic treated group of patients ($P = .0174$ and $P = .0385$, respectively). A similar effect was not observed in the placebo arm (Figure S2).

If we compare bacterial genera dynamic between the treatment arms, the main difference was significant increase in relative abundance of *Bifidobacteria* genera (0.9%, $P = .037$) in intervention arm patients (Table S5). In comparison to the baseline abundance of bacterial genera, we also observed significant *Lactobacillus* and *Subdoligranulum* enrichment in the endpoint stool samples in the treatment group, by 1.88 and 1.32 times, respectively. In the placebo group, the abundance of *Fusicatenibacter*, *Collinsella*, and *Erysipelotrichaceae_UCG-003* group significantly increased by 1.69, 1.56, and 1.31 times, respectively (Figure 2). A similar dynamic of stool microbiota was observed in the intention-to-treat population (Table S6).

At the genus level, differences in beta diversity were assessed using the weighted UniFrac distance and visualized with principal coordinates analysis. Although not statistically significant ($P = .0734$), a tendency toward differences in beta diversity among placebo and synbiotic microbial communities was noticed (Figure S3).

Bacterial Families and Genera Correlated With Clinical Measures

In order to determine the association of specific bacterial taxa with positive effects of synbiotic therapy on uremic toxins and biochemical parameters, a correlation study was performed (Tables S7 and S8). We have not found a significant correlation between IS dynamic and specific changes in the gut microbiome, but changes in serum level

Table 1. Basic Characteristics and Dietary Patterns of 2 Groups of Patients

Variable	Placebo	Treatment	*P* Value
N	17	17	
Gender (male)	9	9	1.000
Comorbidities			
HTN	10	11	.178
DM	4	6	
Other	3	0	
Age (y)	69 ± 8	69 ± 10	.838
BMI (kg/m^2)	25.5 ± 2	26.5 ± 2.7	.245
ACE inhibitors (yes)	14	13	.500
Phosphate binders (yes)	6	8	.728
Proton pump inhibitor (yes)	4	4	1.000
BUN (mmol/L)	11.2 ± 2.2	10.7 ± 1.7	.433
Potassium (mmol/L)	4.7 ± 0.6	4.6 ± 0.8	.708
eGFR (mL/min)	31.3 ± 7.3	26.5 ± 6.5	.062

ACE, angiotensin-converting enzyme; BMI, body mass index; BUN, blood urea nitrogen; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; HTN, hypertensive nephrosclerosis.

of pCS directly correlated with *Prevotella* (rho = 0.428, $P = .012$) and were inversely associated with the *Intestinibacter* genus (rho = –0.476, $P = .004$). A direct correlation was also observed between pCS dynamics and the abundance of *Prevotellaceae* (rho = 0.475, $P = .004$), as well as an indirect one with the *Ruminococcaceae* family (r = –0.369, $P = .032$). Changes in TMAO levels were found to be indirectly related to *Bifidobacterium* (rho = –0.464, $P = .006$) and directly related to Enterococcaceae family abundance (rho = 0.515, $P = .002$).

Estimated GFR improvement was associated with an increase in the relative abundance of *Bifidobacterium* (rho = 0.382, $P = .026$) and *Holdemanella* genera (rho = 0.484, $P = .004$). The abundance of *Faecalibacterium* and eGFR dynamic moved in opposite directions during the study (rho = –0.381, $P = .026$). The hsCRP dynamic was significantly associated with the *Streptococcus genera* and the *Streptococcaceae family* (rho = 0.370, $P = .031$), *Akkermansia genera* and the *Akkermansiaceae family* (rho = 0.362, $P = .035$), *Veillonellaceae* (rho = 380, $P = .027$), and *Bacteroidaceae* (rho = –0.434, $P = .01$) enrichment.

Dietary Factors

Adherence to the diet plan was high, 90% (83–98) in the treatment arm and 91% (84–95) in the placebo arm. There were no significant differences in average dietary intakes of energy, protein, fiber, carbohydrates, and fat between the groups. No significant difference between baseline and endpoint BMI values was observed between groups. The treatment option did not have a significant effect on the BMI dynamic in either group of patients (Table 4).

Gastrointestinal Symptoms and Safety Data

All the 34 patients recruited for the study complained of at least 1 gastrointestinal symptom during the initial GSRS interview, mostly of constipation and indigestion. The posttreatment GSRS interview showed that synbiotic therapy significantly alleviated constipation (Table S9). Two of the patients of intervention arm complained of increased flatulence during synbiotic therapy as a main reason of nonadherence to the study protocol. As mentioned, hemodialysis was initiated in 2 patients in the control group due to hypervolemia. No other safety issues were noted.

Discussion

In comparison to placebo, synbiotic treatment significantly altered levels of IS and pCS in the intervention arm. In comparison to placebo, only the dynamic of total serum IS was significant. Secondary outcomes such as a significant decrease in hsCRP levels and improvement in eGFR were also met. The positive effects of synbiotics were paralleled by a significant shift in microbiome composition.

Up to date, a total of 10 randomized, double-blind, placebo-controlled studies have evaluated the efficacy and safety of prebiotics and probiotics on the level of uremic toxins and inflammatory status (Table S1).[7–16] According to a recent meta-analysis on the subject, prebiotic and probiotic supplementation led to little or no significant effect on levels of uremic toxins.[17,31] As we mentioned, one of the main shortcomings of randomized controlled trials included in these meta-analysis was inadequate control of confounding elements such as diet and antibiotic usage. In a Synbiotics Easing Renal Failure by Improving Gut

Table 2. Effect of Synbiotic Supplementation on Total Serum Levels of Uremic Toxins

Variable	Placebo	Treatment	*P* Value
Baseline IS (μmol/L)	51.1 (36-84)	73.1 (57.1-114)	.114
Endpoint IS (μmol/L)	54.5 (38.2-91.5)	55.3 (44.7-88.1)	.540
ΔIS (%)	6.7 (−0.4 to 16.4)	−21.5 (−11.5 to −34.7)	.000
Baseline pCS (μmol/L)	138.9 (122-152.4)	136.7 (116.7-160)	.946
Endpoint pCS(μmol/L)	134.1 (116.8-153.3)	128 (106.1-149)	.708
ΔpCS (%)	−2.4 (−7.4 to 1.8)	−9.78 (−11.6 to −4.7)	.079
Baseline TMAO (μmol/L)	2.99 (2.25-4.93)	4.33 (3.16-5.23)	.170
Endpoint TMAO (μmol/L)	3.28 (2.4-4.51)	4.13 (3.02-5.04)	.218
ΔTMAO (%)	6.25 (−8 to 27)	−7.6 (−22.4 to 5)	.063

IS, indoxyl sulfate; pCS, p-cresyl sulfate; TMAO, trimethylamine N-oxide.

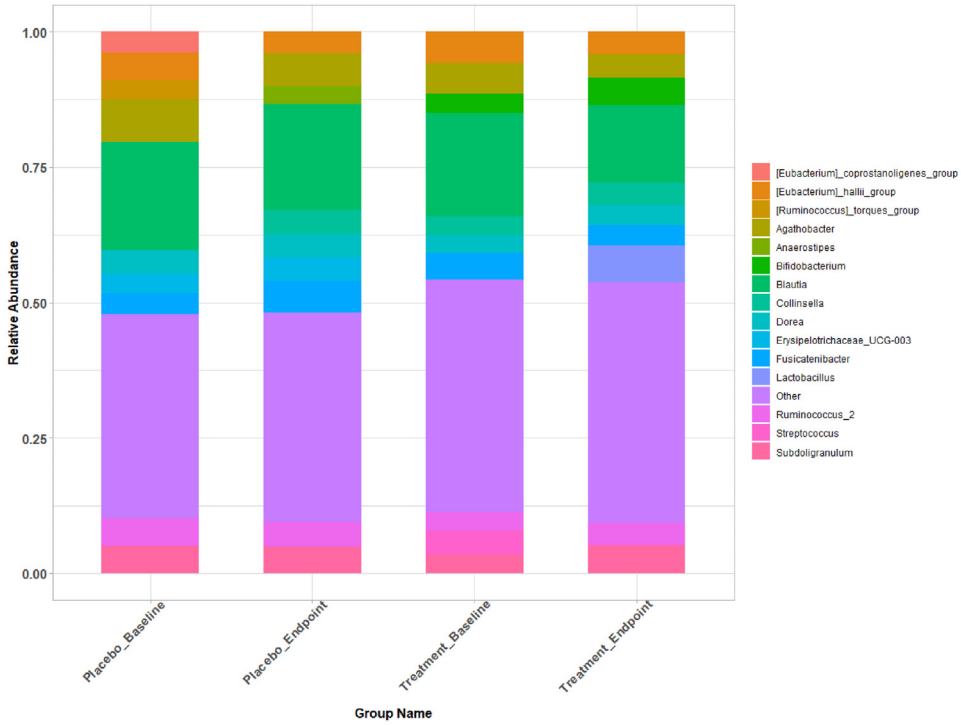

Figure 2. Top 10 bacterial genera, baseline and endpoint relative abundances at the genus level at placebo and symbiotic-treated groups.

Microbiology trial performed by Rossi et al., in which diet and antibiotic usage were strictly controlled, synbiotics had a significant effect on both IS and pCS reduction.[14] Our study, with a protocol very similar to the one in the Synbiotics Easing Renal Failure by Improving Gut Microbiology trial, replicated a similar effect of synbiotic treatment although the impact on pCS levels was less visible.[14] In another study which limited antibiotic usage in study patients, a similar conclusion that prebiotic supplementation decreased total serum IS was also made.[10]

Why are gut-derived uremic toxins so important? Numerous previous studies have shown an important effect of IS, pCS, and TMAO on CV risk and CKD progression. An in-vitro study performed by Muteliefu et al. proved that IS enhanced the activity of nicotinamide adenine dinucleotide phosphate oxidase in cultured vascular smooth muscle

Table 3. The Effect of Synbiotic Supplementation on Biochemical Parameters of Per-Protocol Population

Variable	Placebo	Treatment	*P* Value
Baseline IL6 (pg/mL)	8.9 (5.5-11.3)	9.3 (8.7-12)	.205
Endpoint IL6 (pg/mL)	7.6 (5.4-11.4)	10.6 (7.7-10.7)	.496
ΔIL6 (%)	0.8 (−18.6 to 25.4)	−8.5 (−20.3 to 5.6)	.182
Baseline hsCRP (mg/L)	3.2 (1.8-4.1)	4.3 (3.3-5.8)	.057
Endpoint hsCRP (mg/L)	3 (1.9-4.4)	2.3 (1.9-3.4)	.892
ΔhsCRP (%)	−8.3 (−56.1 to 25)	−39.5 (−49.5 to −25.5)	.001
Baseline albuminuria (mg/g)	213 (117-388)	123 (76.5-332.2)	.375
Endpoint albuminuria (mg/g)	223 (152.2-326)	157 (101.1-252.4)	.259
ΔAlbuminuria (%)	28.3 (−9.3 to 59)	37 (−6.2 to 62.6)	.683
Baseline eGFR (mL/min)	31.3 ± 7.3	26.5 ± 6.5	.062
Endpoint eGFR (mL/min)	32.1 ± 8.5	29.2 ± 6.8	.205
ΔeGFR (%)	8 (−4 to 8.9)	12.5 (7.4-20.3)	.029
Baseline BUN (mmol/L)	11.1 ± 2.2	10.7 ± 1.7	.558
Endpoint BUN (mmol/L)	10.9 ± 2.2	10.9 ± 1.8	.196
ΔBUN (%)	1.1 (−12.1 to 11.4)	0 (−7 to 14)	.683
Baseline potassium (mmol/L)	4.71 ± 0.59	4.58 ± 0.83	.152
Endpoint potassium (mmol/L)	4.83 ± 0.51	4.49 ± 0.52	.929
ΔPotassium (%)	2.4 (−5.3 to 7)	−2.4 (−7 to 6.7)	.357

BUN, blood urea nitrogen; eGFR, estimated glomerular filtration rate; hsCRP, high-sensitivity C-reactive protein; IL-6, interleukin 6.

Table 4. Body Mass Index (BMI) Dynamic and Dietary Factors

Variable	Placebo	Treatment	*P* Value
N	17	17	
Baseline BMI (kg/m^2)	25.5 ± 2	26.5 ± 2.7	.245
Endpoint BMI (kg/m^2)	25.6 ± 2	26.3 ± 2.6	.415
ΔBMI (%)	−18 to 11	−7 to 16	.386
Diet adherence (%)	90 (83-98)	91 (84-95)	
Average daily calorie intake (kCal/kg)	26.4 ± 2.9	28.1 ± 2.2	.064
Carbohydrates (%)	61.3 ± 5.6	58.7 ± 6.2	.208
Fat (%)	30.3 ± 3.2	32.5 ± 3.3	.057
Protein (%)	9.1 ± 1.4	8.5 ± 1.3	.205
Average daily protein intake (g/kg)	0.62 ± 0.08	0.64 ± 0.08	.728
Average daily fiber intake (g/kg)	14.8 ± 1.6	14.2 ± 1.6	.339

cells.[32] That kind of effect may lead to an increase in reactive oxygen species production and muscle cell transformation into osteoblast-like cells and may promote vascular calcification and atherosclerosis during CKD progression.[32] IS has also been implicated in CKD progression, mostly through increased reactive oxygen species production and induction of inflammation and fibrosis. The profibrotic effects of IS are mostly explained by activation of the nuclear factor-$\kappa\beta$ transcription factor.[33] IS is also shown to induce downregulation of Klotho, a gene with aging-suppressive and renoprotective effects.[34] Therefore, any treatment strategy targeting production and clearance of IS, as well as other gut-derived uremic toxins, could have an important clinical effect on the survival of CKD patients and delay the need for renal replacement therapy.

The improvement in eGFR was noted in both groups of our patients, with more pronounced effects in the treatment arm. Although some of the studies with similar subjects showed trends toward improvement or slowing of the decline of renal function in groups treated with probiotics and synbiotics, the meta-analysis of McFarlan et al. did not show significant benefits of synbiotic treatment on renal function (3 studies, 132 participants, mean difference 0.34 mL/minute/1.73 m^2, $P = .79$, $I^2 = 0\%$).[17] A similar conclusion was made in the meta-analysis performed by Jia et al.[31] We must apostrophize that these findings are limited by the risk of bias, imprecision, and high heterogeneity.

High-sensitivity CRP is a liver-derived inflammatory marker with a proven correlation with CV event risk.[35] Our findings are consistent with the findings of a recent meta-analysis.[31,36,37] The meta-analysis of Zheng et al. pointed out great heterogeneity between studies but performed subgroup analysis which showed that probiotics are especially effective in a population with BMI around or under 26 kg/m^2 or if a combination of *Bifidobacteria* and *Lactobacillus* cultures is used.[37] In our study, the effects of synbiotics on hsCRP were not followed by a decrease in IL-6 levels. The meta-analysis of Jia et al., which included 3 studies and 134 patients, even proved that probiotics could possibly increase serum levels of IL-6.[31] That kind of finding should not be perceived as pathologic, as IL-6 may have an important role in the preservation of intestinal wall permeability.[38]

One of the biggest strengths of our study is that it linked the uremic toxins' dynamics with specific changes in microbiome composition. As expected, probiotic therapy increased the relative abundance of *Bifidobacterium* and *Lactobacillus* in the intervention arm. This finding is consistent with the results of only 2 previous studies that analyzed the microbiome shift in probiotic-treated renal patients.[11,14] Although the linear correlation between their abundance and the dynamic of IS and pCS was not observed, the change of microbiome milieu definitely impacted the process of protein fermentation. Similar conclusions have been made by Kano et al. in a study of positive effects of *Bifidobacterium*-fermented milk on serum phenol levels.[39]

Another interesting finding of our study is the increase in relative abundance of the *Subdoligranulum* genus of the *Ruminococcaceae* family in synbiotic-treated patients. *Subdoligranulum* has been mostly mentioned in the context of its association with better blood sugar control and high-density lipoprotein levels.[40] A study performed by Leclercq et al. also pointed out the possible importance of this genus in gut wall permeability in patients with alcohol dependence.[41] The association of *Subdoligranulum* with lower parameters of systemic inflammation was presented by Louis et al.[42] Van Hul et al., on the other hand, hypothesized in their metagenomic study that *Subdoligranulum* is a marker of positive dynamic and greater diversity of the microbiome.[43]

The dynamic in microbiome composition was also observed in our control group. Although the relative abundance of *Fusicatenibacter*, a butyrate-producing bacteria with many positive effects on gut health, increased, that effect was opposed by an increase in *Collinsella* or *Erysipelotrichaceae_UCG-003*, the bacteria that are considered to be proinflammatory.[44-46] We may hypothesize that this is a result of the positive effect of a uniformly prescribed diet and better medication control without support from synbiotics.

The correlation study revealed a direct correlation between the pCS dynamic and *Prevotella_9* abundance and an indirect one with *Intestinibacter* and *Ruminococcaceae*. Lately, several emerging reports have connected *Prevotella_9* existence to low-grade systemic inflammation and several diseases such as rheumatoid arthritis, metabolic states, or even chronic obstructive pulmonary disease.[47] The studies on mice with induced colitis have shown the capability of *Prevotella_9* to stimulate local Th17 lymphocytes to increase local production of proinflammatory cytokines—tumor necrosis factor, IL-6, and IL-8. This could

explain the possible role of *Prevotella_9* in "leaky" gut pathophysiology, with a consequent influx of gut-derived uremic toxins into systemic circulation.[48] *Prevotella_9* is also often associated with constipation and prolonged gut transition time, which could have an effect on gut-derived uremic toxin clearence.[49] *Intestinibacter* has not yet been mentioned in context of renal failure, but its importance for integrity of gut mucosa has been observed in metformin-treated patients.[50] We also observed an inverse correlation between the *Ruminococcaceae* family and the pCS dynamic. *Ruminococcaceae* are a heterogenous bacterial family containing both genera with and without phenol-producing capability, so it is hard to make a conclusion about a possible correlation with uremic toxin production. We can hypothesize that the role of non–phenol-producing genera, such as *Subdoligranulum*, was dominant in our study.

We also observed a positive correlation between *Enterococcaceae* abundance and levels of TMAO. *Enterococcaceae*, with its most prevalent cultured strain *Enterococcus faecium*, are often considered to have an important effect on increased mucosal inflammation and gut permeability in inflammatory bowel disease and lymphocytic colitis.[51] A recent report by Wang et al. reported an effect of *E. faecium* and local inflammation on colorectal carcinogenesis.[52]

Estimated GFR improvement was associated with an increase in the relative abundance of *Bifidobacterium* and *Holdemanella* genera, but the abundance of *Faecalibacterium* and eGFR dynamic moved in opposite directions. *Holdemanella*, a bacterial genus that produces short-chain fatty acids, plays an important role in the preservation of gut mucosal integrity. The abundance of *Holdemanella* is frequently reduced in the gut microbiome of renal failure patients, which may lead to gut barrier instability, increased uremic toxin production, and inflammation that ultimately promote kidney failure progression.[53] One of the controversies of our correlation study is the inverse association between eGFR dynamic and *Faecalibacterium* abundance. *Faecalibacterium*,especially *prausnitzii* subspecies, also known as a next-generation probiotic, plays an important role in gut physiology and human health.[54] Observed correlations have not been described in similar studies, so further investigation on the subject is needed.

Microbiome analysis revealed a link between the *Streptococcus* and *Akkermansia* genera, the *Veillonellaceae* and *Bacteroidaceae* families, and the hsCRP dynamic. Although most of the *Streptococcus* spp. are regular gut commensals with possible beneficial effects, some of the species are known to trigger macrophage activity through Toll-like receptor pathways, driven by immunogenic bacterial products.[55] The reports on the role of *Akkermansia* in the gut health of renal patients are mixed. On one hand, it is known that *Akkermansia* has a positive effect on gut mucosa integrity, but it seems that increased relative abundance of *Akkermansia* follows the progression of CKD, especially in sarcopenic renal patients.[56] A similar point can be said for *Veillonella spp.* of the *Veillonellaceae* family, which is frequently linked to sarcopenia, old age, and frailty.[57] The *Bacteroidaceae* family is a heterogeneous one, mostly depleted in CKD patients. There are no reports of a possible correlation of *Bacteroidaceae* with inflammatory syndrome in renal patients, but its connection with elevated hsCRP in ankylosing spondylitis has been previously described.[58]

GSRS analysis proved that synbiotics significantly ameliorated constipation, one of the most prevalent gastrointestinal symptoms of CKD patients.[59] Although constipation is usually perceived as a benign and minor discomfort, in CKD patients, it is often associated with significant microbiome alteration and increased gut barrier permeability with an effect on systemic inflammation and CV risk.[60,61] Therefore, we must consider the possibility that better clearance of gut-derived uremic toxins with improved gut transit times could have played a part in the observed effect of synbiotic treatment.

Despite the single-center design, a relatively small sample size, and short duration of the study that could have an effect on statistical power for detection of more substantial changes, the main strength of our study is its randomized, double-blind, placebo-controlled design, strict control of antibiotic usage, and diet prescription together with microbiome analysis, which is described in only 2 synbiotic intervention studies of CKD patients to date.

Practical Application

Synbiotic treatment appears to be safe and may lead to a favorable modification of gut microbiome and reduced serum level of IS, increased glomerular filtration rate, and decreased inflammation in patients with CKD. Larger trials are indicated.

Credit Authorship Contribution Statement

Miloš Mitrović: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Visualization, Writing – original draft. **Verica Stanković-Popović:** Funding acquisition, Project administration, Writing – review & editing. **Maja Tolinački:** Data curation, Investigation, Methodology. **Nataša Golić:** Data curation, Investigation, Writing – review & editing. **Svetlana Soković Bajić:** Data curation, Formal analysis, Investigation, Writing – original draft. **Katarina Veljović:** Investigation. **Branislav Nastasijević:** Data curation, Investigation, Methodology. **Ivan Soldatović:** Formal analysis, Project administration, Project administration, Software, Supervision. **Petar Svorcan:** Conceptualization, Writing – review & editing. **Nada Dimković:** Conceptualization, Methodology, Visualization, Writing – review & editing.

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Supplementary Data

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