Probiotics-addicted low-protein diet for microbiota modulation in patients with advanced chronic kidney disease (ProLowCKD): A protocol of placebo-controlled randomized trial

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ARTICLE INFO

ABSTRACT

Microbiota is a term coined to describe the population of bacteria, viruses and fungi that inhabit in symbiosis within a living host. A connection between unbalanced microbiota and chronic kidney disease has been established. In these patients, high levels of urea reach the intestine promoting the overgrowth of bacterial species that are prone to generate uremic toxins. Due to the high morbidity and mortality of this condition, a large number of therapeutic approaches to reduce inflammation and microbial uremic toxins have been proposed, with controversial results. A low protein diet, with a protein intake of 0.6–0.8 g/kg of body weight, is a useful and historically pursued option with this regard. The aim of our study is to evaluate, among patients with advanced renal failure not on dialysis, the synergic beneficial effects of this diet and the selected probiotics *Bifidobacterium longum* (mix DLBL) and *Lactobacillus reuteri* LRE02 (DSM 23878).

1. Introduction

Microbiota is a term coined to describe the population of bacteria, viruses and fungi that inhabit in symbiosis within a living host. The human microbiota is composed of 100 trillion bacterial cells. Bacteroides and Firmicutes together account for 90% (Eckburg et al., 2005; Marchesi et al., 2016).

Microbiota plays a central role in homeostasis, immune system development, organ morphogenesis and functions. Disturbance in microbiota quality and quantity, with a prevalence of pathobionts over the symbionts and eubionts, is defined as "dysbiosis" or "dysbiotic microbiota". This situation may induce gut, lung, liver and kidney diseases, immune system dysfunctions and behaviour alterations (Felizardo, Castoldi, Andrade-Oliveira, & Câmara, 2016; Sommer & Bäckhed, 2013). Healthy microbiota is consistent with beneficial microbial metabolism, as the colonic bacteria participate in food digestion mainly through two catabolic pathways:

a) the saccharolytic one, including bacteria able to ferment carbohydrates and undigested oligosaccharides (prebiotics) turning them into short-chain fatty acids (acetate, propionate and butyrate) with several beneficial effects;

b) the proteolytic one, involving bacteria that perform protein fermentation (putrefaction) generating branched-chain fatty acids, ammonia, amines, indoles, phenols and other toxic agents (Evenepoel, Meijers, Bammens, & Verbeke, 2009; Montemurno et al., 2014).

A connection between unbalanced microbiota and chronic kidney disease (CKD) has been established. In CKD patients, high levels of urea reach the intestine promoting the overgrowth of bacterial species that are prone to generate uremic toxins. Subjects with end-stage renal disease show a significant increase in phyla such as Actinobacteria, Proteobacteria and Firmicutes, whereas healthy people are rich only in Bacteroides and Firmicutes (Vaziri et al., 2013; Wong et al., 2014). In

addition, in CKD subjects the intestinal wall is characterized by the loss of tight junctions and epithelial cell dysfunction. In turn, this higher permeability allows the translocation of bacteria and their fractions into the bloodstream, enhancing the systemic inflammation and accelerated atherosclerosis (Mafra et al., 2014).

Patients with CKD show high prevalence of cardiovascular disease and mortality (Go, Chertow, Fan, McCulloch, & Hsu, 2004; Sarnak & Levey, 2000) due to the traditional (age, hypertension, diabetes, dyslipidemia, smoking) and non-traditional risk factors (high blood volume, anemia, calcium-phosphorus imbalance, vascular calcifications, protein-energy wasting and uremic toxins) (Stenvinkel et al., 2008).

A large amount of uremic toxins is now recognized. In 2003, on the basis of the molecular weight and kinetic behaviour, the European Uremic Toxin Work Group (EUTox) classified 90 retention solutes into three categories: small water-soluble molecules, middle molecules, and protein-bound compounds (Vanholder et al., 2003). In addition to the endogenous metabolism and the external vehicles (food, drugs), dysbiotic microbiota participates to the generation of uremic toxins, through an enhanced proteolytic bacterial metabolism. More specifically, the uremic toxins are produced both from the host and gut microbiota and from the metabolism of food by gut microbiota (Koppe, Fouque, & Soulage, 2018).

P-cresyl-sulphate (PCS) is a protein-bound uremic toxin generated from the transformation of the aromatic amino acids tyrosine and phenylalanine into phenolic metabolites that undergo liver sulfation (Gryp, Vanholder, Vaneechoutte, & Griet-Glorieux, 2017). PCS correlates with the cardiovascular morbidity and mortality in CKD (Lin et al., 2014, 2015; Vanholder, Schepers, Pletinck, Nagler, & Glorieux, 2014) and the progression of renal insufficiency (Wu et al., 2011).

Indoxyl-sulphate (IS) derives from the metabolism of tryptophan into indole and subsequently indoxyl-sulphate in the liver. It is also recognized to be correlated to cardiovascular damage in CKD (15–19).

Lipopolysaccharide (LPS) is a component of the outer bacterial membrane recognized as a strong inflammatory trigger. Increased serum levels of LPS indicate an impaired intestinal wall permeability and dysbiosis (Fuke, Nagata, Suganuma, & Ota, 2019; Mafra et al., 2014).

Lipoprotein-associated phospholipase A_2 (Lp-PLA$_2$) is a serine lipase associated mainly with low-density lipoproteins (LDLs) produced by activated monocytes and macrophages. Lp-PLA$_2$ enters into the vessel wall and catalyses the hydrolysis of the LDLs phospholipids, thus releasing lysophosphatidylcholine and oxidized fatty acids. In this way, the inflammatory cascade is triggered, so inducing the chemotaxis of leucocytes into the sub-intimal space and their conversion into foam cells. Consequently, Lp-PLA$_2$ enhances the growth and the instability of the lipidic core of the atherosclerotic plaques, thus causing acute cardiovascular events (Ballantyne et al., 2004; Cai, Zheng, Qiu, Mai, & Zhou, 2013; Zalewski & Macphee, 2005). Lp-PLA$_2$ is recognized to be a predictor of acute cardiac and cerebral accidents and cardiovascular mortality in general (Ridker, MacFadyen, Wolfert, & Koenig, 2012), diabetic (Hatoum, Hu, Nelson, & Rimm, 2010), dysmetabolic (Persson, Hedblad, Nelson, & Berglund, 2007), and cardiac populations (Maiolino et al., 2012; Robins, Collins, Nelson, Bloomfield, & Asztalos, 2008). The three major heart international societies, the American Heart Association, the American College of Cardiology and the European Society of Cardiology, include the Lp-PLA$_2$ activity in the risk stratification, in order to optimize the lipid lowering treatment (Davidson et al., 2008). Despite this evidence in non-nephropathic subjects (Li et al., 2017; Li et al., 2017), few studies investigated the behaviour of Lp-PLA$_2$ in CKD population (Wang et al., 2016). Dialyzed subjects have Lp-PLA$_2$ levels higher than healthy people correlating with higher cardiovascular morbidity in the early and long-term follow-up (De Mauri, Vidali, Chiarinotti, Bellomo, & Rolla, 2019; Rolla et al., 2015). Since Lp-PLA$_2$ is an inflammatory product and dysbiotic microbiota in CKD can enhance systemic inflammation, there could be some relationship between these molecules. As a matter of fact, the literature agrees that plant-based

diets reduce atherogenic lipids and cardiovascular risk (Dinu, Abbate, & Gensini, 2017; Yokoyama, Levin, & Barnard, 2017) in the general population. In particular, Lp-PLA$_2$ was reduced by 16% after only 4 weeks of a plant-based diet in dysmetabolic patients with normal renal function (Najjar, Moore, & Montgomery, 2018).

Due to the high morbidity and mortality, a large number of therapeutic approaches to reduce inflammation and microbial uremic toxins has been proposed, with controversial results. The direct removal of the toxins from blood and tissues through oral adsorbents has demonstrated poor results (Lee et al., 2014). The main intervention remains the reduction of the generation of these toxins by re-shaping the dysbiotic microbiota. We defined this approach as "microbial modulating therapy", and in particular we identified three different but complementary interventions, also named as the three "P": protein, prebiotic and probiotic.

Among CKD patients a low protein diet (LPD), with a protein intake of 0.6–0.8 g/kg of body weight and mainly from vegetables, is a useful and historically pursued option to reduce uremic symptoms, hypertension, hyperphosphatemia, proteinuria, cardiac complications, malnutrition and to delay the progression of renal failure towards the end stage (Cupisti et al., 2018; Riccio, Di Nuzzi, & Pisani, 2015). In addition, it is a safe and low-cost therapy, and reduces morbidity and mortality (Piccoli et al., 2016; Rysz, Franczyk, Cialkowska-Rysz, & Gluba-Brzózka, 2017). Several studies confirmed that LPD associated with adequate fibre intake counteracts the dysbiosis and reduces the uremic toxins (Black et al., 2018; Marzocco et al., 2013).

Prebiotics are defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or limited number of bacterial species already resistant in the colon, and thus attempt to improve host health" (Gibson & Roberfroid, 1995). They are found in many fruits, vegetables, cereals, and stimulate the growth of bacterial strains with prevalent saccharolytic pathway (Gibson et al., 2017; Valcheva & Prebiotics, 2016). Several studies demonstrated that prebiotics such as inulin, galactooligosaccharides, dextrins and whole grains could reduce the serum levels of uremic toxins, particularly PCS and IS in CKD (Dinu et al., 2017; Rossi, Klein, Johnson, & Campbell, 2012), and restore a proper intestinal wall permeability with a consequent reduction of LPS (Fuke et al., 2019).

Probiotics were defined in 1989 as "live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). The use of probiotics alone in CKD subjects failed to modulate the microbiota, while a combination of pre- and probiotics, mainly fructo-oligosaccharides (FOS) with Bifidobacterium or Lactobacillus spp., may have some benefits in reducing the uremic toxins (Fuke et al., 2019; Koppe et al., 2018; Pavan, 2016; Rossi et al., 2014; Simeoni et al., 2019; Vaziri, 2016).

Despite a large amount of studies, literature still lacks clinical trials evaluating the synergic outcome of a low protein diet in combination with selected probiotics in reducing microbial and inflammatory uremic toxins and in preserving residual renal function in patients with advanced renal failure not on dialysis.

2. Aim

The aim of the Probiotics-addicted Low-protein diet in Chronic Kidney Disease (ProLowCKD) protocol is to evaluate, among patients with advanced renal failure not on dialysis, the synergic effects of low protein diet and selected probiotics.

2.1. Primary outcome measures

Reduction from baseline of the microbial inflammatory uremic toxins

- *A significant* reduction from baseline of the concentration of

proteolytic microbial groups;

- *A significant* increase from baseline of the concentration of saccharolytic microbial groups;
- Reduction *of more than 20%* from baseline of the serum concentration *of total and free p-cresyl sulphate (t-PCS and f-PCS) and of total and free indoxyl sulphate (t-IS and f-IS),* as markers of dysbiotic microbiota;
- Reduction *of more than 20%* from baseline of the serum concentration of LPS, as a marker of altered intestinal barrier permeability;
- Reduction *of more than 20%* from baseline of the serum concentration of Lp-PLA$_2$ as a marker of cardiovascular disease.

2.2. Secondary outcome measures

Delay of the decline of Glomerular Filtration Rate (GFR) and amelioration of the Quality of Life (QoL)

- Reduction *of GFR* from baseline *of more than 20%*;
- Haemoglobin, bicarbonate, C-reactive protein (CRP), parathormone (PTH) and urinary protein excretion *in the range usually accepted for CKD population, according to the guidelines;*
- *A significant* increase from baseline of fat-free body mass (kg) and Hand Grip strength (kg);
- *A significant* reduction from baseline of total body water (TBW) and fat mass (kg);
- *A significant* increase from baseline of *Short Form-36 (SF-36)* questionnaire scores.

3. Study design

ProLowCKD is a single-centre, double-blind, placebo-controlled, randomised study. At enrolment (T0) participants were prescribed an LPD in addition to their ongoing pharmacological therapy and according to their comorbidities; after 2 months (T2) they were randomized in accordance to a 1:1 ratio to receive probiotics or placebo for other 3 months (T5) in addition to the continuation of LPD. Enrolled subjects were invited to assume two doses of probiotic/placebo for 1 month and one dose for 2 months.

Randomization was performed on a 1:1 basis to receive odd- or even envelopes, according to the odd- or even registration number at enrolment.

Neither the clinician nor the patient knew the content of the odd- and even envelopes.

The evaluations were performed according to the following schedule:

At enrolment (T0): nephrological evaluation; illustration of protocol and signature of the informed consent; delivery of the SF-36 questionnaire; dietary and dietitian counselling and body composition evaluation by bioimpedentiometry (see "Nutritional assessment"); blood biochemical analysis: haemoglobin, urea, creatinine, mean urea and creatinine clearance, GFR estimation, sodium, potassium, uric acid, calcium, phosphate, PTH, acid-base balance, CRP, albumin, PCS, IS, Lp-PLA$_2$, LPS; 24 h-urine biochemical parameters: urea, creatinine, sodium, proteins; stool microbial analysis.

Urine nitrogen excretion (UNN) was calculated according to the following formula: UNN = urinary urea (g/day) + (0.031*body weight), and the total UNN according to the formula: TUNN = UNN + 0.625* urinary proteins (g/day).

At the beginning of the study a dosage of vitamin D was always provided in order to adapt the supplementation therapy, in accordance to the clinical guidelines for CKD patients.

After two months (T2): nephrological evaluation; dietitian counselling and body composition evaluation by bioimpedentiometry (see "Nutritional assessment"); blood biochemical analysis and 24 h-urine biochemical parameters as above; urine nitrogen excretion (UNN) and the total UNN; stool microbial analysis.

Randomization 1:1, as described above

After additional 3 months (T5): nephrological evaluation; delivery of the SF-36 questionnaire; dietitian counselling and body composition evaluation by bioimpedentiometry (see "Nutritional assessment"); blood biochemical analysis and 24 h-urine biochemical parameters as above; urine nitrogen excretion (UNN) and the total UNN; stool microbial analysis.

4. Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki (2000) of the World Medical Association.

The study was approved by the Ethical Committee of Our Institution (215/CE n. CE 30\17) on March 13th, 2017. On April 27th, 2018 an extension of the study, including the quantification of serum LPS, was approved by the Ethical Committee of Our Institution.

Every patient enrolled signed an informed consent.

The trial has been registered on ClinicalTrials.gov with the number NCT04204005.

The data will be published in anonymous form.

5. Population

The trial recruited adult subjects with advanced chronic renal failure not yet on dialysis.

Inclusion criteria: patients with age from 18 to 80 years, afferent to our Nephrology and Dialysis Department; estimated glomerular filtration rate (eGFR: CKD-EPI equation) lower than 25 ml/min/1.73 m^2, without acute kidney impairment; ability to provide an informed consent.

Exclusion or drop out criteria: patients with previous renal transplantation or chronic inflammatory bowel disease; refusal to sign the informed consent, or refusal of the LPD; any antibacterial therapy during the T2-T5 period; starting of dialysis; death or voluntary drop out.

6. Intervention

Low protein diet composition: protein load 0.6 g/kg of body weight/day, energy intake 30–35 kcal/kg/day, salt less than 6 g/day, phosphorus load less than 800 mg/day, low content saturated fats and cholesterol, high content of fibres; calcium, vitamin D, folic acid, vitamin B12, iron, erythropoietin supplementation according to the usual clinical indications. Adherence to the diet was tested through dietary interview and urine nitrogen excretion according to the Maroni-Mitch formula (D'Alessandro, Piccoli, Calella, & Brunori, 2016).

Prebiotics were provided together with the LPD in the light of its high content of plant-based foods.

Probiotics composition: 5×10^9 of *Bifidobacterium longum* (mix DLBL), 1×10^9 *Lactobacillus reuteri* LRE02 (DSM 23878) and maltodextrin (total 2 g). The probiotic species employed were granted the Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) in 2007. The specific amount of viable probiotic cells used was in accordance with the International Guidelines and also with our previous human clinical results involving phylogenetically related beneficial bacteria. No previous human trials have been conducted with either the mix DLBL or the *L. reuteri* LRE02 strain.

Placebo composition: Maltodextrin (2 g).

The subjects were directed to consume the treatments in the morning and/or in the evening on an empty stomach after dissolution in a glass of water at room temperature.

The sachets containing the two different formulations were identical in appearance. They were only distinguished by a unique randomisation code on each box containing 30 sachets.

The scientific rationale at the basis of probiotics selection is mainly attributed to the evidence of their presence in centenarians and the

ability to synthesize a significant amount of reuterin, a natural antibiotic molecule with a large spectrum of action.

In this way, *L. reuteri* LRE02 may provide a valuable contribution to the reduction of uremic toxins produced mainly by the putrefactive fraction of the gut microbiota.

Moreover, a previous study conducted by our group showed that all the *B. longum* bacteria in the DLBL mix have a similar cytokines modulation profile, with a remarkable increase of IFN-γ, whose effects on innate immunity are thoroughly documented (Drago, Toscano, Rodighiero, De Vecchi, & Mogna, 2012; Nicola et al., 2016).

7. Quality of Life

Quality of Life was assessed by the *SF-36* questionnaire, which has been validated and widely used in nephropathic subjects (Cukor et al., 2012; Ware, Snow, Kosinski, & Gandek, 1993). It was administered at T0 and T5.

The SF-36 consists of eight scaled scores, which are the weighted sums of the questions in their respective sections. Each scale is directly transformed into a 0–100 scale on the assumption that each question carries equal weight. The higher the score, the higher the disability. Since nephropathic subjects may present depressive symptoms, probably due to the awareness of their chronic condition, particular attention was paid to the psychological and emotional domains of SF-36 (limitations due to health problems, limitations due to personal or emotional problems, emotional well being, social functioning, general health perception). *A psychological counseling was made available* for patients with poor SF-36 score, depressive symptoms or directly requiring a psychological evaluation.

8. Nutritional assessment

Dietary counselling. A qualitative and quantitative "24-hour recall" interview was employed to estimate the previous intake of proteins, lipids, carbohydrates and calories and to assess the dietary habits in order to optimize and personalize the LPD.

Physical exam to measure body weight, height, BMI (kg/m^2), middle upper arm circumference (cm), Triceps Skinfold (mm) of the left arm using skinfold caliper "Holtain Tanner", dominant Hand Grip strength (kg) using Hydraulic Hand Dynamometer Ownor's Manual (Sammons Preston), according to the reference values (Frisancho, 1990; World Health Organisation, 1995).

Bioelectrical impedance analysis (BIA) to estimate total body water (TBW), fat-free body mass (kg), fat mass (kg), phase angle through an Akern model 101 (Akern Srl).

9. Laboratory analysis

For the simultaneous quantitative assay of both total and free p-Cresyl Sulphate and total and free Indoxyl Sulphate in human serum a high-performance liquid chromatography technique coupled with tandem mass spectrometry *was* employed (B.S.N. Srl, Castelleone (CR) Italy).

The quantitative determination of Lp-PLA$_2$ activity *was* performed through the new PLAC® test (Diazyme Laboratories, Inc. 12,889 Gregg Court, Poway, CA 92,026 USA) in Clinical Chemistry Laboratory, Department of Health Sciences, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy.

Our Institution does not currently perform Lp-PLA$_2$ analysis in clinical practice. Anyway, the PLAC® test could be easily implemented in the laboratory routine on ADVIA® 1800 Clinical Chemistry Analyzer (Siemens Healthcare Diagnostics) at the cost of about 30€ / sample. Since it could predict the cardiovascular risk and estimate the efficacy of the nutritional intervention, the savings will be re-calculated in terms of patient care.

Routine laboratory measurements (haemoglobin, urea, creatinine, mean urea and creatinine clearance, GFR estimation, sodium, potassium, uric acid, calcium, phosphate, PTH, acid-base balance, CRP, albumin) were performed on ADVIA® 1800 Clinical Chemistry Analyzer (Siemens Healthcare Diagnostics) in Clinical Chemistry Laboratory, Department of Health Sciences, Amedeo Avogadro University of Eastern Piedmont, Novara, Italy.

Plasma LPS concentration was measured with the LAL Chromogenic Endpoint Assay (Hycult Biotechnology, Uden, The Netherlands) according to manufacturer's instructions. LPS concentration was expressed in pg/ml and calculated based on a standard curve.

Fecal samples were collected according to the protocol and stored at −20 °C until analysis. The 16S rDNA will be sequenced using the Next Generation Sequencing (NGS) by the Ion Torrent® equipment (Thermo Fisher Scientific, Monza, Italy). Selected microbial phyla will be quantified, namely: Firmicutes, Proteobacteria, Actinobacteria, Bacteroides; families: Enterobacteriaceae and Clostridiaceae; genera/species: *Escherichia coli*, Clostridium, Bifidobacterium, Lactobacillus, Bacteroides, Eubacterium, and Peptococcus.

Storage. The serum samples were collected in tubes and stored in controlled access refrigerators in Nephrology and Dialysis Unit and/or in Clinical Chemistry Laboratory, Novara (Italy). The clinical data were collected in a controlled access computing database in Nephrology and Dialysis Unit, Novara.

The faecal samples were collected and stored in controlled access refrigerators at Probiotical Research Srl (Novara, Italy).

10. Statistical analysis

In order to optimize the methods, we performed preliminary tests in our Laboratory on samples from subjects similar to the enrolled patients, showing a mean t-PCS equal to 93 ± 30 μM.

Using the software (piface.jar) and assuming a reduction of 30% in serum concentration of t-PCS before and after the P/P intervention with a power of 95% and alpha error equal to 5%, we calculated a sample size population of 50 patients equally divided into two groups.

Data will be reported as mean ± standard deviation. Comparison will be performed using the Student's *t*-test for paired and unpaired data. A p less than 0.05 will be considered statistically significant. Statistical analyses will be performed by SPSS statistical software v.17.0 (SPSS Inc., Chicago, IL, USA).

11. Preliminary results

We analyzed data from the first 22 (14 male, age 63 ± 14 years) patients with complete follow-up. At the enrolment (T0) the renal function, measured as the mean of creatinine and urea clearance, and CKD-EPI equation was 20.3 ± 7.1 and 18.1 ± 4.1 ml/min, respectively, and did not differ after 2 and 5 months. During the follow-up we did not observe any difference in the haemoglobin, blood urea nitrogen (BUN), uric acid, albumin, calcium, phosphorus, total, HDL and LDL cholesterol, C- Reactive Protein (CRP) and PTH levels. A decrease in triglycerides levels, even if not statistically significant, was observed after the first two months of LPD (239 ± 210 vs. 172 ± 71 mg/dl) (Table 1).

At T0, patients were investigated about their dietary habits: 55 ± 12 g/kg/day of proteins (0.8 g/kg/day; range 0.4–1.1) and 23 ± 6 Kcal/Kg/day. The total urine nitrogen and protein catabolic rate (PCR) significantly decreased at T2 compared to baseline: 11.4 ± 3 vs. 9.7 ± 2.6 g/24 h/kg and 71.4 ± 18.8 vs. 60.9 ± 16.2 g/24 h, respectively, then remaining stable at T5 (Table 1).

At T2, Lp-PLA$_2$ significantly decreased (170.2 ± 52 *vs.* 158.5 ± 51.9 nmol/ml/min, p = 0.04). The serum concentrations of uremic toxins were 10 times higher than in general population and remained stable.

After the randomization, we analyzed data from patients assuming odd (OP) and even (EP) numbered disposables to test the probiotic or

Table 1
List of biochemical parameters tested.

	T0	T2	T5	p
Clearance (ml/min)	20.3 ± 7.1	18.5 ± 5.4	19 ± 7.5	NS
EPI (ml/min)	18.1 ± 4.1	19 ± 4.3	18.6 ± 6.6	NS
Urine protein (g/24h)	1.4 ± 1.6	1.5 ± 2	1.8 ± 2.5	NS
TUNN (g/24h/kg)	11.4 ± 3	9.7 ± 2.6*	10 ± 3	*0.007 T0vsT2
PCR (g/24h)	71.4 ± 18.8	60.9 ± 16.2^	62 ± 18.6	^0.007 T0vsT2
Hb (g/dl)	12.7 ± 1.8	12.4 ± 1.8	12.4 ± 1.8	NS
BUN (mg/dl)	48.1 ± 15.8	42.7 ± 12.7	45 ± 17.8	NS
Uric acid (mg/dl)	6.4 ± 1.4	6.2 ± 1.4	5.9 ± 1.4	NS
Albumin (mg/dl)	4.2 ± 0.3	4.1 ± 0.4	4.2 ± 0.4	NS
Ca (mg/dl)	9.2 ± 0.5	9 ± 0.6	8.8 ± 0.5	NS
P (mg/dl)	3.6 ± 0.8	3.5 ± 0.6	3.6 ± 0.6	NS
Total cholesterol (mg/dl)	193 ± 18	183 ± 41	194 ± 39	NS
HDL cholesterol (mg/dl)	44 ± 15	45 ± 14	46 ± 12	NS
Triglycerides (mg/dl)	239 ± 210	172 ± 71	176 ± 98	NS
LDL cholesterol (mg/dl)	111 ± 43	103 ± 37	116 ± 34	NS
CRP (mg/dl)	0.5 ± 0.6	0.5 ± 0.7	0.5 ± 0.5	NS
HCO_3 (mEq/l)	23 ± 4	24 ± 3	24 ± 3	NS
PTH (ng/ml)	73 ± 53	85 ± 52	101 ± 54	NS
$Lp\text{-}PLA_2$ (nmol/ml/min)	170.2 ± 52	158.5 ± 51.9§	162.9 ± 40.2	§0.04 T0vsT2
t-PCS (µM)	134.23 ± 69.9	117.68 ± 71.95	116 ± 74.26	NS
f-PCS (µM)	5.1 ± 2.9	4.1 ± 3.26	5.46 ± 5.72	NS
t-IS (µM)	31.4 ± 15.5	31.43 ± 14.63	35.23 ± 17.8	NS
f-IS (µM)	1.54 ± 0.68	1.46 ± 0.84	1.66 ± 0.7	NS

placebo (P/P) treatment.

At T5 OP showed a significant higher renal function (OP: 21.5 ± 6.1 *vs.* EP: 15.7 ± 6 ml/min, p = 0.03).

t-PCS significantly decreased in EP after two months of LPD (155.7 ± 73.6 *vs.* 115.1 ± 77.1 µM, p = 0.05) but not in OP (Table 2).

t-IS, that was only marginally higher in EP at T0, slightly increased in EP after the randomization: at T5, EP had significantly higher t-IS than OP (43.4 ± 20.9 vs 26.9 ± 8.7, p = 0.02). A similar trend was recognized for f-IS, where the difference between the two groups was significant at T5 (EP 1.9 ± 0.17 vs OP 1.3 ± 0.5 µM, p = 0.03) (Table 2).

No side effects due to the LPD and/or P/P were observed.

12. Conclusions

A double-blind, randomized, placebo-controlled clinical trial is ongoing with the aim to evaluate the synergic effects of a low protein diet (LPD) associated with selected probiotics in reducing the microbial inflammatory uremic toxins.

As the safety of gut microbiota exerts a selective impact on the progression of kidney and systemic disease, our position is that the therapeutic approach should be holistic as well and aimed to re-

modulate the dysbiotic and proteolytic microbiota in favour of the eubiotic and saccharolytic components. This positive effect would in turn reduce the generation of uremic toxins.

A limitation of our study is the lack of testing of a wider range of microbial toxins: for instance, the trimethylamine N-oxide (TMAO), generated from choline and carnitine by gut microbial metabolism, is largely correlated with atherosclerosis and cardiovascular disease and would be probably a good marker for therapeutic interventions (Ramezani, Massy, & Meijers, 2016; Velasquex, Ramezani, Manal, & Raj, 2016).

The nutritional approach was hereby named as "microbial modulating therapy" and our interventions were focused on the three "P" (Protein, Prebiotics and Probiotics), very useful to concretize the shift from proteolytic to saccharolytic metabolism. In addition, when conducted and controlled by skilled clinicians, the nutritional therapy is safe and devoid of side effects, assuring high physical performance and quality of life.

Finally, the nutritional therapy is a low-cost treatment, if compared with pharmacological or dialytic approach.

In conclusion, we aim to identify a new role for microbial modulating therapy in the management of advanced renal failure, as both a supportive treatment and kidney-friendly lifestyle.

CRediT authorship contribution statement

Andreana De Mauri: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Project administration, Writing - original draft, Writing - review & editing. **Deborah Carrera:** Methodology. **Marco Bagnati:** Resources. **Roberta Rolla:** Resources. **Doriana Chiarinotti:** Methodology, Supervision. **Luca Mogna:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing, Funding acquisition. **Marco Pane:** Resources. **Angela Amoruso:** Resources. **Mario Del Piano:** Conceptualization, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review & editing, Funding acquisition.

Table 2
Comparison of microbial uremic toxins between OP (odd) and EP (even) numbered disposables at the different time points.

11/11 patients		T0	T2	T5	p
t-PC (µM)	OP	112.8 ± 61.9	120.3 ± 70	105.4 ± 58.3	*0.05 EP2 vs EP0
	EP	155.7 ± 73.6	115.1 ± 77.1*	126.7 ± 89	
f-PC (µM)	OP	4 ± 1.9	3.3 ± 1.7	4.4 ± 3.1	NS
	EP	6.2 ± 3.3	4.8 ± 4.3	6.5 ± 7.5	
t-IS (µM)	OP	26.9 ± 13.1	26.8 ± 7.1	26.9 ± 8.7	^0.02 OP5 vs EP5
	EP	35.8 ± 14.9	36 ± 18.8	43.4 ± 20.9^	
f-IS (µM)	OP	1.2 ± 0.4	1.2 ± 0.3	1.3 ± 0.5	§0.03 OP5 vs EP5
	EP	1.8 ± 0.8	1.6 ± 1.1	1.9 ± 0.17§	
$Lp\text{-}PLA_2$ (nmol/ml/min)	OP	175 ± 64	161 ± 65	163 ± 41	NS
	EP	164 ± 37	156 ± 37	162 ± 42	

Declaration of Competing Interest

Luca Mogna, Marco Pane and Angela Amoruso are employees of Probiotical Research Srl.

Mario Del Piano is the Head of Clinical Research of Probiotical SpA.

Acknowledgements

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