Nephrology

In Vitro and *In Vivo* Assessment of Intraintestinal Bacteriotherapy in Chronic Kidney Disease

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Chronic kidney disease may progress to end-stage renal disease, which requires dialysis or kidney transplantation. No generally applicable therapies to slow progression of renal disease are available. Bacteriotherapy affords a promising approach to mitigate uremic intoxication by ingestion of live microbes able to catabolize uremic solutes in the gut. The present study evaluates the nonpathogenic soil-borne alkalophilic urease-positive bacterium *Sporosarcina pasteurii* (Sp) as a potential urea-targeted component for such "enteric dialysis" formulation. Data presented herein suggest that Sp survives through exposure to gastric juice retaining the ability to hydrolyze urea. *In vitro*, 10^9 cfu (colony forming units) of Sp removed from 21 ± 4.7 mg to 228 ± 6.7 mg urea per hour, depending on pH, urea concentration, and nutrient availability. Beneficial effects of Sp on fermentation parameters in the intestine were demonstrated *in vitro* in the Simulator of the Human Intestinal Microbial Ecosystem (SHIME) inoculated with fecal microbiota. Enumeration of marker organisms suggested that presence of Sp does not disturb microbial community of the SHIME. Additionally, a pilot study in 5/6th nephrectomized rats fed 10^9 cfu of live Sp daily throughout the study demonstrated that the tested regimen reduced blood urea-nitrogen levels and significantly prolonged the lifespan of uremic animals. *ASAIO Journal* 2006; 52:70–79.

Renal insufficiency, often a consequence of multisystem disorders such as hypertension, diabetes, or atherosclerosis, has a broad prevalence afflicting millions of people worldwide. One consequence of declining renal function is impaired excretion of nitrogenous solutes resulting in retention of potentially harmful substances, termed uremic toxins and defined as compounds that (1) under normal conditions are excreted by kidneys; (2) accumulate progressively with the decrease of renal function; and (3) have negative impact on physiologic and biochemical functions.[1] When diseased kidneys are no longer able to maintain life, the disorder is termed end-stage renal disease (ESRD). Approximately 400,000 Americans with ESRD are under treatment, most commonly by thrice-weekly hemodialysis.

The National Kidney Foundation characterized the stages of progressive kidney disease according to the level of residual renal function measured as glomerular filtration rate (GFR).[2] For patients with GFR 20–50/min (estimated from the serum creatinine concentration), who are considered to be "predialysis," few US Food and Drug Administration–approved medicines are available, each targeting a single uremic solute such as potassium, phosphate, and water. Appreciating the reality that no effective treatment is available for predialysis patients (except the use of angiotensin-converting enzyme/angiotensin II receptor blocker inhibitors as a mild kidney-protecting modality for patients with late-stage chronic kidney disease) and the expense of treating the next stage of renal function loss by hemodialysis (approximately $65,000 annually per patient), the search for alternative strategies focused on delaying and/or preventing the need for dialysis is a vital economic and medical concern. Additionally, the large population of individuals with lesser degrees of renal insufficiency might also benefit from a kidney "stabilizing" regimen.

The concept of "enteric dialysis" as an alternative strategy for solute extraction in kidney failure is based on the fact that the intestinal wall functions as a semipermeable membrane. Driven by concentration gradient, solutes with elevated concentration in circulating blood diffuse from plasma into the lumen and a large portion of uremic solutes are differentially distributed throughout the bowel.[3,4] Uremic solutes might be extracted by binding to ingestible solute-specific sorbents within the gut,[3] but this approach requires that large quantities of each sorbent be ingested daily. An innovative "enteric" approach to mitigate uremia using live bacteria that, when ingested, catabolize uremic solutes in the gut has been tested recently[5–7] and is further investigated in the present study.

Azotemia, the accumulation of nitrogenous waste products, chiefly urea, in the blood, is the hallmark of renal failure. Urea is the predominant nitrogen waste product of protein catabolism.[8] Proteins contain approximately 16% nitrogen by weight, and during protein catabolism, virtually all of the nitrogen is converted to urea. Less than 19% of protein nitrogen is converted to substances other than urea, mainly creatinine and uric acid. Pre- and postdialysis urea concentration is a current

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marker to evaluate adequacy of dialysis. Two urea clearance parameters—URR (urea reduction ratio) and Kt/V, a dynamic parameter that evaluates the fall in urea concentration during dialysis—are inversely related to the odds of a dialyzed patient's death.[9,10] Although mechanisms involved in urea's toxicity are poorly understood,[11] it is well established that urea contributes to the synthesis of other toxic moieties including guanidines and carbamylation products.[12,13] Moreover, excess urea induces generation of free radicals and oxidative stress, which are implicated in cellular damage.[14]

In the present study, a soil-borne spore-forming ureolytic gram positive bacterium, *Sporosarcina pasteurii* (Sp), was evaluated as a urea-targeted component for bacteriotherapy of azotemia. Although Sp is not an indigenous inhabitant of the human intestine, it has been isolated from human feces and is considered nonpathogenic. Urea hydrolase (urease) constitutes about 1% of the dry weight of this bacterium[15] and is expressed constitutively, in contrast to many other ureolytic bacteria in which synthesis of urease is induced by substrate, environmental pH, or nitrogen starvation.[16,17] Moreover, Sp can use urea as a sole nitrogen source, and according to some reports, needs urease only to generate a preferable nitrogen source, ammonia. We analyzed urea hydrolysis by Sp cells *in vitro,* in batch culture experiments simulating conditions encountered by ingested bacteria in the gastrointestinal tract environment, and in the Simulator of Human Intestinal Microbial Ecosystem (SHIME, see Methods section for description),[18,19] where it shows effects beneficial for colon microbiota. In 5/6th nephrectomized rats, we demonstrate that feeding with Sp attenuates blood urea-nitrogen levels (BUN), slows the progression of azotemia, and increases in the life span of uremic animals.[20,21]

Materials and Methods

Media and Growth Conditions

Sporosarcina pasteurii strain 6452 (formerly classified as *Bacillus pasteurii*) was obtained from American Type Culture Collection. To prepare inoculums, Sp was cultivated at 37°C in tryptic soy broth (TSB) (Becton, Dickinson, Sparks, MD) adjusted to pH 9.0. Either exponentially growing culture or freeze-dried aliquots of an exponentially growing culture were used as indicated. For the preparation of freeze-dried samples, cells were collected by centrifugation at 5,000 rpm for 10 minutes and resuspended in TSB medium supplemented with 10% skim milk at cell density 10^9 cfu/ml. One-milliliter aliquots were distributed into cryogenic vials and frozen at –70°C. Frozen aliquots were dried overnight in a lyophilizer Freeze Mobile 12 (Virtis Company, Gardiner, NY). Cell counts in initial culture, frozen samples, and freeze-dried formulation were determined by the most probable number (MPN) method in TSB. No loss in cell viability was observed, and all samples retained 10^9 cfu/ml of viable cells. In all experiments, freeze-dried cells were rehydrated in physiological saline for 30 minutes before inoculation.

For preparation of spores, Sp was cultivated for 2–3 weeks on tryptic soy agar (TSA) pH 9.0 at 37°C, harvested, washed in sterile distilled water, resuspended in water, and pasteurized for 10–15 minutes in an 80°C water bath. Spores that survived pasteurization were collected by centrifugation and viable spore count per gram of wet pellet was determined by MPN method in TSB pH 9.0. Pellets were stored at 4°C.

Both TSB and artificial intestinal fluid (AIF) were used to measure urea hydrolysis in batch culture experiments. Both media were adjusted to the indicated pH and supplemented with filter-sterilized urea to final concentration of 100, 200, or 300 mg/dl. In some experiments, creatinine (15 mg/dl), uric acid (15 mg/dl), and ammonium chloride (100 mg/dl, or 18.7 mM) were added to the medium, as indicated. Standard AIF adjusted to pH 7.5 ± 0.1 was prepared as described in the *U.S. Pharmacopeia*.[22] Modified AIF (AIF M2) was used in some *in vitro* experiments. AIF M2 was prepared by supplementing AIF with 10% DeMan, Rogosa, Sharpe broth (MRS, standard medium for growing lactic acid bacteria), dextrose, yeast extract, and $NiCl_2$ to final concentration of 1%, 0.3%, and 100 μM, respectively, with pH being adjusted as indicated.

Measurement of Growth, Urea Hydrolysis, Ammonia Liberation, Creatinine, and Uric Acid Concentrations

Growth of Sp in all experiments was evaluated by reading the optical density of the culture at 600 nm (OD_{600}) using Spectrophotometer V-530 (Jasco, Easton, MD). Urea-nitrogen concentration[23,24] was determined using standards and reagents supplied with the Blood Urea Nitrogen Reagent Kit (535, Sigma, St. Louis, MO). Urea hydrolysis was monitored by comparing urea-nitrogen concentrations in bacterial supernatants and the corresponding control medium incubated in the same conditions. Data are expressed as percent of control.

Ammonia liberation via urea hydrolysis by the pure urease was compared to that of the Sp culture as follows. Three milligrams (approximately 582 units) of partially purified urease derived from Sp (U-7127; Sigma) or 300 mg of freeze-dried Sp cells were added to two sterile 200 ml screw cap bottles containing 100 ml standard AIF fortified with100 mg/dl urea and 1 g/dl pancreatin. A third bottle was used as a control. All bottles were sealed and placed in a water bath at 37°C and shaken at 100 rpm. Calibrated ammonia ion-selective electrode Accumet 13-620-505 (Fisher Scientific, Pittsburgh, PA) was used to monitor ammonia production continuously in real time.

Creatinine and uric acid concentrations in culture supernatants and control media were measured using direct end-point procedure with Creatinine or Uric Acid Reagent sets (Medicos, S. Plainfield, NJ) according to the manufacturer's instructions.

Gastric Juice Stability

Survivability of vegetative cells and spores in gastric juice were tested in artificial gastric fluid pH 1.4 prepared according to *U.S. Pharmacopoeia*.[20] Vegetative cells in the exponential growth phase or spores were washed in physiologic saline (0.8% NaCl with 0.1% Bacto peptone), inoculated into artificial gastric fluid at initial density of 10^7 cfu/ml, and incubated at 37°C for up to 3 hours. Postexposure viable cell counts were determined at 30-minute, 1-hour, 2-hour, and 3-hour intervals by MPN method in TSB medium pH 9.0 and by plating aliquots from serial dilutions on TSB pH 9.0 agar plates.

SHIME Study

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME, Ghent University, Belgium) consisted of five double-

Table 1. Description of the Different Vessels of the SHIME Culture System and Their Operational Parameters

Vessel	Intestinal Segment	Volume (l)	Retention Time (h)	pH
1	Stomach	0.2	2	2.0–2.5
2	Small intestine	0.3	6	5.0–6.0
3	Colon *ascendans*	0.4	18	5.5–6.0
4	Colon *transversum*	0.8	36	6.0–6.4
5	Colon *descendans*	0.5	22	6.6–6.9

jacketed vessels maintained at a temperature of 37°C.[18,19] Each vessel simulated the conditions characteristic of a particular part of the human gastrointestinal ecosystem (**Table 1**). The first two vessels, representing stomach and small intestine, worked as a fill-and-draw system, whereas the last three, representing the colon, were continuously stirred tank reactors with a total retention time of 74 hours. The pH controllers maintained the pH within fixed limits by automatic addition of 0.1 M HCl or 0.1 M NaOH. There was no gas exchange between vessels, and the headspace was flushed with oxygen-free N_2 twice a day for 15 minutes to ensure anaerobic conditions.

The inoculum of human microbiota was prepared by collecting 10 g freshly voided fecal samples from three healthy volunteers. Each sample was diluted in 100 ml phosphate buffer (0.1 M pH 7.0) containing 0.1% sodium thioglycolate as a reducing agent. Samples were homogenized and centrifuged 1 minute at 500*g* to remove particulate material. The supernatants were pooled and 50 ml was inoculated into each of the last three vessels. The system was stabilized over a period of 2 weeks by the addition of 200 ml fresh carbohydrate-based medium containing 0.1% arabinogalactan, 0.2% pectin, 0.3% starch, 0.04% glucose, 0.3% yeast extract, 0.1% peptone, 0.4% mucin, and 0.05% cystein to the first vessel of the culture system three times a day.[18] To simulate stomach acidification, the pH of feed was gradually decreased to 2.0. The passage of food in the small intestine was simulated by addition of 100 ml simulated bile liquid [6 g/l oxgal (Difco, Bierbeek, Belgium), 0.9 g/l pancreatin (Sigma, Bornem, Belgium), and 12.6 g/L $NaHCO_3$] to the next vessel, vessel.2 Steady-state conditions in the system were assessed by monitoring short-chain fatty acids (SCFA) and ammonium concentration. Liquid samples were collected and frozen at –20°C for subsequent analysis. SCFA were extracted from samples with diethyl ether and measured with Di200 gas chromatography (GC, Simadzu Hertogenbosch, Netherlands). GC was equipped with a capillary free fatty acid-packed column (EC-1000Cap (Alltech, Laarne, Belgium) 25 × 0.53 mm; film thickness 1.2 mm), a flame ionization detector, and a Delsi Nermag 31 integrator (Thermo Separation Products, Wilrijk, Belgium). Nitrogen was used as a carrier gas at a flow rate of 20 ml/min. Column temperature was set at 130°C, while temperatures of the injector and detector were set at 195°C. For ammonium measurement, ammonium in samples was liberated as ammonia by the addition of an alkali MgO using the 1026 Kjeltec Auto Distillator (FOSS Benelux, Amersfoort, Netherlands). Released ammonia was distilled from samples into boric acid solution, and the resulting solution was back-titrated with 665 Dosimat and 686 Titroprocessor (Metrohm, Berchem, Belgium).

Test-run of the SHIME consisted of three periods. After inoculation of the reactor with a large intestinal microbiota from healthy individuals, the ecosystem was stabilized for 2 weeks with normal SHIME nutrition supplied (start-up period). During the 2-week treatment period, 10^9 cfu of live Sp cells was added to vessel 2 (small intestine) twice a day at the time of feeding. To test whether fermentation parameters return to start-up levels, treatment period was followed by control period, when supplementation of Sp was discontinued and the SHIME was fed with normal nutrition for additional 2 weeks.

To monitor the bacterial ecosystem in the SHIME, enumeration of fecal marker organisms was performed twice a week by plating 0.1 ml aliquots from three serial dilutions of the SHIME suspension in 0.85% NaCl on appropriate selective medium (see **Table 2**).[18] Plates were incubated either aerobically or anaerobically at 37°C for 24–72 hours. For *Lactobacillus* and *Bifidobacterium sp.*, anaerobic incubation was performed in jars with 84% N_2, 8% CO_2, and 8% H_2 gas atmosphere adjusted by the Anoxomat 8000 system (Mart, Sint-Genesius-Rode, Belgium).

Animal Study

A blinded pilot study using a rat model of chronic kidney disease (CKD) was conducted to evaluate *in vivo* application of Sp in uremia. Eighteen Sprague-Dawley rats (nine male and nine female, weight 265–320 g) from Charles River Labs had 5/6th two-step nephrectomies performed at CRL. After the surgery, animals were transported to Thomas Jefferson University, where the study was conducted. Rats were fed with 110900 AIN-93 M Purified Rodent Diet (Dyets, Bethlehem, PA) free of yeast and other microbes. All animals were screened for serum creatinine concentration (Scr), weight, and sex. Twelve rats with the highest Scr were placed into two matched groups. Two-sided analysis of variance with the Bonferroni correction applied showed no significant difference in Scr, BUN, and body weight between groups at baseline (**Table 3**). Starting at day 21 after surgery, the diet in group 1 (Sp) was supplemented with pellets containing 1×10^9 cfu of frozen vegetative Sp cells mixed with 5 g feed. Group 2 received just 5 g feed in addition to regular regimen. Supplements were hand-fed to the animals once a day throughout the study. Rats were caged individually and monitored for approximate total feed intake and probiotic pellets consumption. Body weight, Scr, and BUN were measured every 4 weeks as indicated. A control group of seven nonnephrectomized rats (Scr = 0.2 ± 0.07) received the same food with no supplements. No control animals died during the study (Scr at the end of the study = 0.4 ± 0.1). Days of survival, BUN, and body weight were the primary endpoint variables. The endurance of this study consisted of 156 days (from days the rats were received at Thomas Jefferson University to the day of death, by exposure to CO_2). All rats were necropsied to confirm that contralateral kidney had been removed. All procedures involving animals were performed according to the guidelines of the Institutional Animal Care and Use Committee.[25]

Limited analysis of fecal microbiota was performed for rats fed with Sp and for the placebo group. Fresh fecal samples were obtained from individual animals at baseline (before the treatment) and after 8 weeks of feeding. Samples were placed into 5 ml Para-Pak Enteric Plus Transport System medium (Meridian Bioscience, Cincinnati, OH) and stored at –20°C for

Table 2. Microbial Counts (log cfu) in Vessels 3, 4, and 5 of the SHIME Reactor during Start-up (n = 4), Treatment with *S pasteurii* (n = 4) and Control Period (n = 4)

Microbial Group (Medium)	Period	Vessel 3	Vessel 4	Vessel 5
Total aerobes	Start-up	8.39 ± 0.52	8.52 ± 0.08	8.21 ± 0.27
(Brain heart infusion agar)	Treatment	8.39 ± 0.46	8.06 ± 0.26*	8.29 ± 0.26
	Control	8.19 ± 0.20	8.51 ± 0.26	8.31 ± 0.21
Total anaerobes	Start-up	8.29 ± 0.69	8.1 ± 0.35	8.15 ± 0.58
(Brain heart infusion agar)	Treatment	8.01 ± 0.53	7.46 ± 0.81	7.92 ± 0.69
	Control	7.83 ± 0.54	7.82 ± 0.62	7.70 ± 0.58
Lactobacillus sp.	Start-up	7.33 ± 0.25	7.18 ± 0.24	7.12 ± 0.38
(Rogosa agar)	Treatment	7.04 ± 0.56	6.96 ± 0.51	6.96 ± 0.47
	Control	6.52 ± 0.30†	6.20 ± 0.27†‡	6.60 ± 0.72
Enterococcus sp.	Start-up	7.04 ± 0.45	6.73 ± 0.44	6.26 ± 0.47
(Enterococcus agar)	Treatment	6.83 ± 0.32	6.66 ± 0.28	6.48 ± 0.53
	Control	6.45 ± 0.37	6.39 ± 0.42	6.43 ± 0.31
Escherichia coli	Start-up	7.33 ± 0.35	6.82 ± 0.46	6.88 ± 0.17
(McConkey agar)	Treatment	7.51 ± 0.29	7.43 ± 0.41	7.18 ± 0.59
	Control	7.26 ± 0.19	7.34 ± 0.15	7.09 ± 0.11
Bifidobacterium sp.	Start-up	6.72 ± 1.19	6.65 ± 1.21	6.38 ± 0.90
(Raffinose Bifido agar)	Treatment	5.71 ± 0.38	5.70 ± 0.32	5.88 ± 0.53
	Control	6.06 ± 1.21	6.43 ± 0.68	6.74 ± 0.56
Staphylococcus sp.	Start-up	6.23 ± 0.71	5.76 ± 0.44	5.94 ± 0.62
(Mannitol Salt agar)	Treatment	6.52 ± 0.27	6.23 ± 0.24	6.17 ± 0.49
	Control	6.17 ± 0.29	5.21 ± 1.53	5.60 ± 1.14
Fungi	Start-up	3.74 ± 0.23	3.48 ± 0.45	3.84 ± 0.20
(YPD agar)	Treatment	3.19 ± 0.69	2.98 ± 0.19	3.54 ± 0.14
	Control	4.04 ± 0.00	3.38 ± 0.67	3.33 ± 0.77
Clostridium sp.	Start-up	8.27 ± 0.53	8.27 ± 0.08	8.26 ± 0.27
(Azide agar)	Treatment	8.24 ± 0.26	7.91 ± 0.22*	8.07 ± 0.42
	Control	8.17 ± 0.52	8.09 ± 0.42	8.10 ± 0.12

Data are mean ± SD.
* Significantly different from the start-up period, $p \leq 0.05$.
† Significantly different from the start-up period, $p \leq 0.01$.
‡ Significantly different from treatment period, $p \leq 0.05$.

analysis.[26] Fecal mass was re-suspended in physiological peptone saline at approximate concentration of 0.02 g/ml. Serial dilutions in saline were prepared, and 0.1 ml aliquots from dilutions 10^{-5} and 10^{-7} were plated on MRS agar to obtain *Lactobacillus sp.* counts and TSB pH 9.0 agar plates to enumerate the population of Sp. Plates were incubated at 37°C for 48 hours. MRS plates were kept in anaerobic BBL GasPak Pouches (Beckton Dickinson, Sparks, MD). The dilutions that had more than 50 colonies per plate were taken into account. Final counts are presented as log cfu per gram of feces.

Results

In Vitro Analysis

Human intestine contains a variety of nitrogenous compounds, most of which can be used by bacteria as nitrogen sources. To test the ability of Sp to catabolize urea in the presence of other nitrogen sources, we initially tested Sp live vegetative cells for utilization of nitrogenous wastes in peptide-based medium supplemented with urea, creatinine, uric acid, and ammonium chloride (TSB+, **Figure 1**). Growth curves

Table 3. Survival, Body Weight, and BUN and Scr Concentrations in Control Rats, Rats Fed with *S pasteurii*, and Placebo

Group	Period	Survival (no. of rats)	Body weigh (g)	BUN (mg/dl)	Scr (mg/dl)
Control	Baseline	7 (4m/3 f)	305.7 ± 14.8	12.0 ± 0.7	0.20 ± 0.03
	4 weeks	7	458.0 ± 45.5	13.0 ± 2.2	0.20 ± 0.03
	8 weeks	7	541.0 ± 63.0	15.0 ± 0.8	0.40 ± 0.04
	16 weeks	7 (4m/3 f)*	558.0 ± 67.8	16.0 ± 3.3	ND
Placebo	Baseline	6 (3m/3 f)	265.5 ± 14.5	65.2 ± 6.9†	1.20 ± 0.20‡
	4 weeks	6 (3m/3 f)	326.5 ± 28.7	55.5 ± 12.0†	0.98 ± 0.19‡
	8 weeks	4 (2m/2 f)	374.8 ± 48.5	99.8 ± 30.2†	1.48 ± 0.36‡
	16 weeks (mean ± SD)	2 (1m/1 f)*	436.0 ± 80.0†	104.0 ± 10.0‡§	ND
S pasteurii	Baseline	6 (3m/3 f)	286.5 ± 20.2	64.3 ± 17.4†	0.80 ± 0.07†
	4 weeks	6 (3m/3 f)	390.7 ± 52.5	47.8 ± 7.3†	0.70 ± 0.06†
	8 weeks	6 (3m/3 f)	440.3 ± 62.4	62.0 ± 10.4†	0.87 ± 0.12†
	16 weeks	4 (1m/3 f)*	423.0 ± 65.1	59.5 ± 25.2†	ND

Data are mean ± SEM for the surviving animals unless otherwise indicated. m, males; f, females, ND, .
* Number of surviving animals up to the day the animals were killed.
† Significantly different from control group, $p < 0.05$.
‡ Significantly different from control group, $p < 0.01$.
§ Significantly different from baseline, $p < 0.05$.

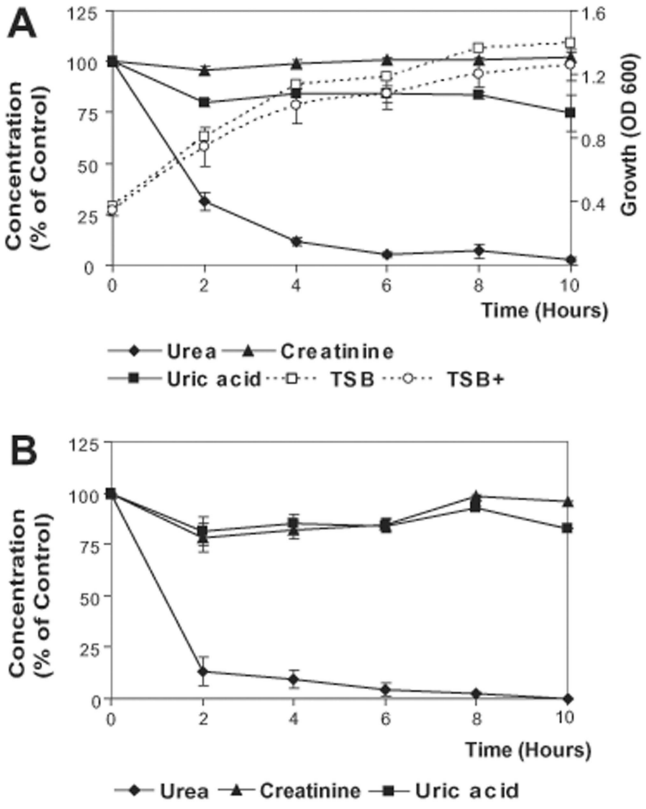

Figure 1. Urea, creatinine, and uric acid concentration profiles in *Sporosarcina pasteurii* cultures in TSB+ pH 9.0 (A) and AIF pH 7.5 (B). (A) 10^8 cfu of live cells were inoculated into 100 of TSB pH 9.0 (TSB) or TSB pH 9.0 supplemented with 100 mg/dl urea, 15 mg/dl creatinine, 15 mg/dl uric acid, and 40 mg/dl of ammonium chloride (TSB+). (B) 10^8cfu of vegetative cells were inoculated into 100 of fortified AIF pH 7.5 supplemented with 100 mg/dl urea, 100 mg/dl NH_4Cl, 15 mg/dl creatinine, and 15 mg/dl uric acid and incubated at 37°C for up to 10 hours. To limit aeration and simulate an anaerobic intestinal environment in these experiments, cultures were placed in plastic containers with almost no head space and were not placed on the shaker. Sister tubes without bacterial inoculum served as controls. Data presented are mean ± SEM from three independent experiments.

presented in **Figure 1A** show that bacteria grew well in both TSB and TSB+, suggesting that ammonium, creatinine, uric acid, and urea did not have significant effect on the growth rate at pH 9.0 ($p > 0.5$ by Student t test, n = 3). After only one generation (2 hours of incubation) in TSB+, about 70% of urea was removed from medium, indicating that Sp can catabolize urea in the presence of ammonium and other nitrogen sources. After 2 hours, the rate of urea hydrolysis decreased, suggesting that it may be dependent on urea concentration in the medium. At 8 hours, only 4 mg/dl urea (4% of initial concentration) was still present. In these conditions, Sp did not catabolize creatinine, because no significant changes in the levels of creatinine were observed in the bacterial culture compared to control media (**Figure 1A**). A slight decrease in uric acid concentration was observed in supernatants of bacterial cultures and was not registered in the control medium. Although this effect can be attributed to the utilization of uric acid by Sp, another possible explanation would be precipitation of urate which has been reported to occur in alkaline solutions within 24 hours.[27] Hydrolysis of urea by Sp results in alkalinization of

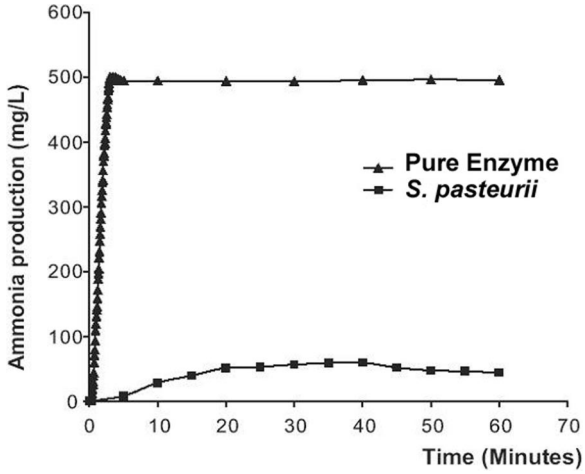

Figure 2. Profiles of ammonia production by purified urease and vegetative Sp cells.

environment and may cause precipitation of urate. Because samples for analysis were taken from the culture supernatant, this will cause the decrease in uric acid concentration.

To confirm the above observations in conditions simulating gastrointestinal tract environment, similar experiments (**Figure 1B**) were performed in AIF pH 7.5 without pancreatin, generally accepted as a standard for simulated intestinal fluid (*U.S. Pharmacopoeia*). In these studies, no nutrients were provided other than urea, ammonium chloride, uric acid, and creatinine. Although within the duration of the experiment, no growth of Sp was observed (data not shown, n = 3), 10^8 cfu still removed urea with a rate similar to, if not greater than, that observed in a growing TSB+ culture within the first two hours (**Figure 1A**). No significant effects on levels of creatinine and uric acid were detected in AIF after 10 hours of incubation, confirming the previous observation that Sp does not catabolize creatinine and uric acid under the conditions tested. For this reason, creatinine and uric acid were not supplemented in further experiments.

End products of urea hydrolysis, carbon dioxide, and ammonia may disturb the intestinal microbial community. To evaluate ammonia production we measured ammonia liberation by 300 mg of freeze-dried vegetative Sp cells in standard AIF pH 7.5 supplemented with 100 mg/dl urea and 1 g/dl pancreatin and compared it with pure enzyme preparation (**Figure 2**). The corresponding amount of urease (3 mg) was calculated based on the fact that urease constitutes about 1% of the dry weight of Sp.[15] Sp hydrolyzed 100% of urea in 1 hour and less than 5 mg/dl ammonia was produced (**Figure 2**). When compared with pure enzyme, which hydrolyzed the urea in 5 minutes, liberating 50 mg/dl ammonia, it is evident that Sp efficiently hydrolyzed urea and produced less ammonia than pure urease. In these experiments, AIF contained 1 g/dl pancreatin, which complicated evaluation of growth by direct OD measurement due to the turbidity. For that reason, growth of Sp was evaluated by plating aliquots of culture diluted serially in TSB. No significant increase in cfu was registered during the experiment (n = 2).

Ureolytic rates for bacterial cells in their natural environment generally depend on a number of factors including urea concentration, cellular levels of urease, and transport rates for

urea and ammonium ion.[15] In Sp, which lacks urea permease and active urea transport,[28] the rate of urea hydrolysis is dependent on the rate of urea diffusion across plasma membrane and hence is determined by the concentration of substrate in medium.[29] Urea hydrolysis rate by purified Sp urease increases with increasing urea concentrations and pH,[15] but whole-cell ureolysis has not been characterized in detail. Environmental parameters such as ammonium and urea concentrations and pH exert profound effects on membrane physiology, energy metabolism, and growth of Sp.[30,31] In alkaline conditions with high urea concentrations, ammonium is necessary for a sufficient alkalinization of the cytoplasm, which requires urease activity and occurs through the diffusion of NH_3 and an influx of NH_4^+ into the cell.[30] Concentration of ammonium in human colonic effluent varies within a narrow range between 14 and 20 mM[32,33] that is lower than physiological range (40 mM) for growing Sp cells.[30] In the ascending colon, where bacterial fermentation is most active, pH is mainly acidic and varies within the range between 5 and 7.5. Other parameters, such as nutrient supply and temperature in the intestine, also may affect the ability of Sp to hydrolyze urea. Considering these differences between natural Sp environmental and conditions in the human intestine, it was important to verify that Sp retains the ability to remove urea in conditions, which simulate colon. These questions were addressed in separate experiments. To simulate the fed state nutrient supply of human intestine, AIF M2 was used. The amount of urea hydrolyzed in one hour was measured in TSB+ pH 7.5, TSB+ pH 5.0, and AIF M2 supplemented with 100 mg/dl urea and 100 mg/dl ammonium chloride at pH 5.0 and 7.5 and compared with that in TSB+ at pH 9.0 (**Figure 3A**). No significant difference in whole-cell ureolysis was observed between pH 7.5 and pH 9.0 in TSB+ (75.4 ± 3.4 and 67.5 ± 2.1, respectively, n = 3, $p = 0.23$) and between AIF 5.0 and TSB 5.0 (24.9 ± 5.9 and 19.7 ± 6.2, n = 6, $p = 0.48$). Maximum rate of urea hydrolysis was observed in AIF 7.5 and was 20–30% higher than in TSB 7.5 and TSB 9.0 ($p < 0.005$ and $p < 0.05$, respectively) and four or five times higher than that at pH 5.0 ($p < 0.001$). In this condition, 10^9 cfu of Sp removed almost 100% of urea (94.3 ± 3.9, n = 6) within the first hour. Although the rate of hydrolysis was significantly reduced at pH 5.0, 10^9 cfu of freeze-dried Sp completely removed 100 mg of urea within only 4 hours (n = 3, not shown).

Urea concentration in the blood of uremic patients can rise to 460 mg/dl with a mean concentration of 230 ± 110 mg/dl.34 To further evaluate the ureolytic capacity of Sp, we analyzed urea depletion in AIF M2 and TSB+ at pH 5.0 and 7.5 and urea concentrations 100, 200, and 300 mg/dl (**Figure 3B**). These conditions simulate *in vitro* the range of pH, urea concentrations, and nutrient supply in the intestine of uremic patients. No growth was registered in all conditions tested but Sp cells were still able to extract urea from solution, confirming our previous observation that urea hydrolysis in Sp is not growth-dependent. The average amount of urea hydrolyzed (milligrams per hour) was dependent mostly on urea concentration and pH and varied from 21.3 ± 4.7 (AIF M2 100 mg/dl urea, pH 5.0, n = 3) to 228.3 ± 6.7 (AIF M2, 300 mg/dl urea, pH 7.5, n = 6) milligrams per hour. The amount of urea hydrolyzed in the first hour was lower under all conditions tested suggesting physiologic adjustment of cells to their new environment.

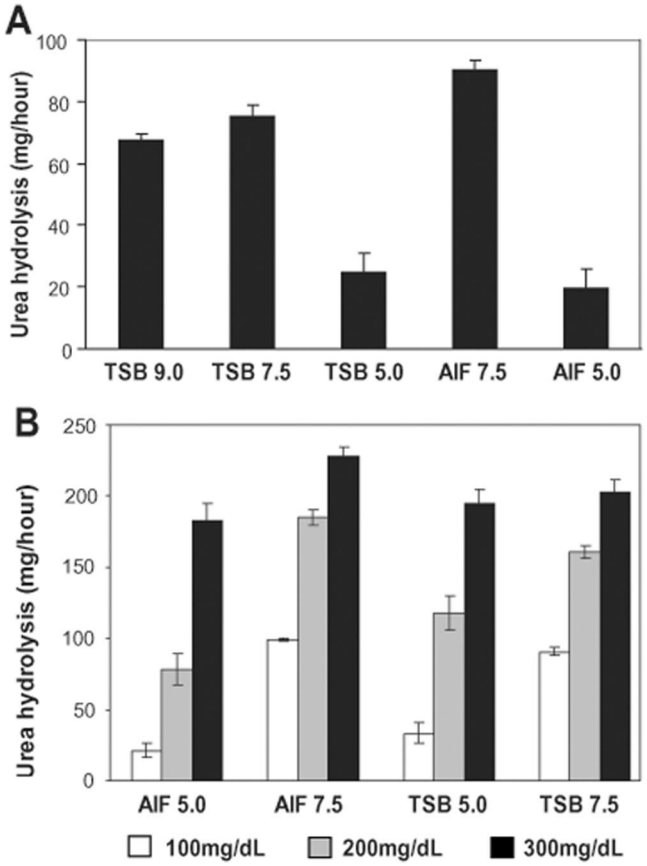

Figure 3. Comparison of rates of urea hydrolysis by 10^9 cfu of freeze-dried Sp in different conditions. (A) Dependence of amount of urea hydrolyzed in 1 hour on the pH in TSB+ and AIF M2. 10^9 cfu of lyophilized Sp was inoculated into 100 of each medium and urea-nitrogen concentration was measured 1 hour later. (B) Effects of concentration of urea and pH on rate of urea hydrolysis by whole-cell urease in TSB+ and AIF M2. In these experiments, 100 of medium was inoculated with 10^9 cfu of freeze-dried Sp. After each 1-hour interval cells were collected by centrifugation, aliquots from the supernatant were analyzed for urea-nitrogen concentration and cells were immediately resuspended in fresh 100 of the same medium. The amount of urea (milligrams per hour) removed from the medium after each hour of incubation (up to 5 hours) was estimated as a difference in urea-nitrogen content between supernatant and appropriate control. All media were also supplemented with 100 mg/dl of ammonium chloride, a concentration typically present in the human colonic effluent as a result of bacterial degradation of urea and amino acid deamination.[30] Data are mean ± SEM from three to six independent experiments.

To reach the large intestine and perform ureolysis, ingested Sp cells have to pass through the body's natural defense system, gastric juice. Low pH in the stomach kills most of the ingested pathogenic bacteria with high efficiency and protects the body from their invasion. Although the general requirement for probiotic bacteria is survival at pH 2–3 (fed-state gastric acidity) for 3 hours,[35] we chose to evaluate the survivability of Sp in fasting state gastric conditions, pH 1.4.[36] As could be expected, spores were more tolerant to these harsh conditions: only 10-fold reduction in viable cfu was observed for spores after 1 hour compared with 100-fold reduction for vegetative cells (n = 3). After 2 hours of incubation 5×10^5cfu

Table 4. Concentration of Short-Chain Fatty Acids and NH_4^+ (μM/g suspension) in Vessels 3, 4, and 5 of the SHIME Reactor during Start-up (n = 7), Treatment with *S pasteurii* (n = 10), and Control Period (n = 10)

Parameter	Period	Vessel 3	Vessel 4	Vessel 5
Acetic acid	Start-up	27.5 ± 6.3	33.4 ± 2.6	38.8 ± 3.5
	Treatment	32.1 ± 6.0	33.0 ± 2.4	37.7 ± 3.6
Control		25.0 ± 5.0*	29.5 ± 2.1†‡	35.4 ± 3.8
Propionic acid	Start-up	6.6 ± 2.7	10.7 ± 1.8	8.15 ± 1.0
	Treatment	8.7 ± 1.7	12.4 ± 0.7§	7.92 ± 0.9
Control		7.5 ± 1.6	12.2 ± 0.9§	7.70 ± 1.0
Butyric acid	Start-up	0.2 ± 0.1	6.6 ± 2.9	7.9 ± 0.5
	Treatment	1.1 ± 1.1§	9.7 ± 0.6†	8.3 ± 2.9§
Control		0.4 ± 0.1*†	9.5 ± 1.1†	9.0 ± 1.1§
Other acids	Start-up	0.1 ± 0.0	1.4 ± 0.8	1.9 ± 0.4
	Treatment	0.2 ± 0.2§	2.1 ± 0.2§	2.2 ± 0.1§
	Control	0.1 ± 0.1*	1.1 ± 0.3‡	1.1 ± 0.3†‡
Total acids	Start-up	34.4 ± 5.4	52.1 ± 4.4	61.0 ± 3.6
	Treatment	42.0 ± 8.7	57.1 ± 3.3*	61.4 ± 4.4
	Control	33.0 ± 6.5*	52.3 ± 2.8‡	58.4 ± 3.8
Ammonium	Start-up	11.4 ± 6.3	21.6 ± 2.6	23.1 ± 3.5
	Treatment	14.4 ± 6.0	25.2 ± 2.4†	26.8 ± 3.6†
	Control	12.1 ± 5.0	23.9 ± 2.1	26.7 ± 3.8§

Data are mean ± SD.
* Significantly different from treatment period, $p \leq 0.05$.
† Significantly different from the start-up period, $p \leq 0.01$.
‡ Significantly different from treatment period, $p \leq 0.01$.
§ Significantly different from the start-up period, $p \leq 0.05$.

were recovered from spore suspension compared to only 4×10^4 cfu from the suspension of vegetative cells (20 and 250-fold reduction in viable cfu, respectively; n = 3). No further decrease in viability was registered in spore suspension, while in cell suspension the count continued to decrease resulting in only 10^3 viable cfu after 3 hours of incubation.

SHIME Study

To evaluate the possible effects of Sp feeding on the normal microbial community and fermentation parameters of the human intestine, the SHIME reactor study was performed. The enumeration of marker organisms in vessels 3, 4, and 5 (**Table 2**) indicated that the microbial population of the SHIME was not dramatically altered by Sp and remained unchanged throughout the study interval. However, some differences persisted during the treatment in vessel 4 (colon *transversum*), where a decrease in total aerobes (about threefold) and *Clostridia sp.* (about 2.3-fold) was registered compared with the start-up period. Both effects were transient and counts returned to start-up levels 2 weeks after the termination of the treatment. Supplementation with Sp also affected the *Lactobacilli* population in the colon: significant decreases in *Lactobacilli* counts were observed in both vessels 3 and 4 during the treatment period (about twofold). This effect developed through the control period, bringing the counts down up to 10-fold compared with the start-up period at the end of the run (**Table 2**).

Fermentation parameters measured in the SHIME included three major short-chain fatty acids (acetic, propionic, and butyric acids) and other short-chain fatty acids, total acids, and ammonium, which represent products of dissimilatory metabolism of amino acids and are physiologically active in host tissues. Start-up molar ratio of propionate: acetate: butyrate registered at the end of start-up period in the SHIME (**Table 4**) indicated that fermentation parameters typical of normal colon environment had been established during the stabilization period in vessels 3, 4, and 5.[37] Overall, feeding with Sp had some significant effects on fermentation parameters in the SHIME. The production of acetic acid decreased in both vessels 3 and 4 after the treatment was terminated, during the control period. The increase in propionic acid was observed in vessel 3 during the treatment period and persisted through the control period. An increase in butyric acid production was detected in all three vessels during treatment but persisted only in vessels 4 and 5. Production of other short-chain fatty acids (caproic, isocaproic, valeric, and isovaleric acids) also increased in all three vessels within the treatment period but returned to baseline (start-up) levels after treatment was terminated. Significant transient increase in total acids, registered in vessel 4, also correlated with Sp supplementation. The significant effects observed in vessel 4 indicate a generally higher fermentative activity in this vessel and suggest that Sp proliferates mainly in colon *transversum.*

The production of ammonium increased in both vessel 4 and 5 during treatment period (17% increase compared with the start-up period). In vessel 5, this effect lasted through the control period, indicating that the effect of Sp may persist for at least 2 weeks after the termination of feeding. An apparent correlation between Sp feeding and rise in ammonia production was observed in vessel 4, suggesting that added bacterium was mainly active in the colon *transversum*. Overall, these data indicate a definite fermentative effect of Sp with the positive effects on levels of major short-chain fatty acids, propionic and butyric.

Animal Study

The rat model of CKD is generally used to evaluate the effect of the proposed treatment on kidney function. In this model, 5/6th of kidney tissue is removed surgically leaving an animal with about 15% of kidney function upon the recovery from

Table 5. Percent Change in Body Weight and BUN from Baseline to Time of Death in the Control, Placebo, and Sp Groups

Group	% Change	Mean	SEM	Median
Control	Body weight (7)	51	7.9	51
(n = 7)	BUN (7)	29	6.3	29
Placebo	Body weight (6)	–3	9.0	–8
(n = 6)	BUN (4)	120	5.8	118
Sp	Body weight (6)	25	4.4	22
(n = 6)	BUN (6)	4	26.7	–16

operation. In the present study, this model was used to evaluate a possible use of Sp in bacteriotherapy of azotemia.

Differences and/or trends were observed between control, placebo, and Sp-fed animals (**Tables 3 and 5**). As was expected, animals which had undergone nephrectomy developed renal insufficiency with BUN and Scr levels at baseline significantly higher than those in control group (**Table 3**, $p < 0.05$ for BUN and $p < 0.001$ for Scr). Average BUN levels in the placebo group seemed to increase gradually with time and in surviving animals at 16 weeks were 5–6.5 times higher ($p < 0.001$) than BUN levels in the control group and about 1.5 times higher than baseline BUN ($p < 0.05$). This may indicate that azotemia in operated animals is progressing with time. Scr levels in the placebo group did not show significant increase over baseline, although this observation may be attributed to the fact that Scr levels were not measured at the end of the study (week 16) due to technical difficulties (**Table 3**).

Feeding with 10^9cfu of Sp daily attenuated the increase in BUN levels. In the placebo group, average BUN levels increased by 120 ± 5.8% (**Table 5**) from baseline to time of death, indicating progression of azotemia in nephrectomized animals. BUN levels in the Sp group remained elevated in comparison with levels in the control group ($p < 0.01$), but were stable throughout the feeding period (**Table 3**) with no significant changes from baseline to time of death (**Table 5**, $p < 0.01$, percent change 4 ± 26.7, n = 6). BUN data suggest that Sp slows the progression of azotemia observed in the placebo group.

Feeding with Sp also prolonged the lifespan of uremic rats by at least 22.3% (Student *t* test, $p < 0.05$), increasing the average survival time after surgery from 115.8 ± 16.02 days (placebo, mean ± SEM, n = 6) to 148.5 ± 5.7 days (Sp, mean ± SEM, n = 6).

Significant differences ($p < 0.05$) were observed between the placebo and Sp groups in percent change in body weight from baseline to time of death (**Table 5**). Percent change in the Sp group (25 ± 4.4, n = 6) was lower than in the control group, but significantly higher than in the placebo group (-3.4 ± 9.0, n = 6), suggesting a beneficial effect of Sp on the general health and/or nutritional status of nephrectomized animals.

Interestingly, the effect of Sp was gender-dependent, with more profound effect in females. At the end of the study, the surviving females in the Sp group (100% survival, compared with 33% survival in the males in the Sp group, and 33% in the females in the placebo) had BUN levels of 35 ± 8.23 mg/dl, which were significantly lower than at baseline (64.2 ± 17.4) and much lower than that found in a single surviving female in the placebo group (94 mg/dl) (**Table 3**). The only surviving male out of the three treated with Sp for 16 weeks had a BUN level of 133 mg/dl, higher than at baseline (64.2 ± 17.4) and similar to that found in the only surviving male in the placebo group (114 mg/dl). Taken together, these data suggest that Sp attenuates BUN levels in uremic animals, with a more profound effect in females. Additional studies are required to establish the positive difference in males and females.

Baseline serum creatinine levels in nephrectomized animals, in both the placebo and Sp groups, were about five times higher ($p < 0.001$) than in control rats, indicating uremic state. A slight decrease in Scr in Sp-fed animals as compared with animals in the placebo group was not significant (**Table 3**).

Limited analysis of fecal microbiota in rats fed with placebo and Sp revealed no effect of Sp on the population of *Lactobacilli sp.* in the rat intestine. Although effect of Sp on *Lactobacilli* population in the colon was registered in the SHIME, there was no significant difference between baseline *Lactobacilli* counts in the Sp group (log cfu/g feces 8.24 ± 0.97) and counts after 8 weeks of treatment (log cfu/g feces = 8.36 ± 0.86) in uremic rats. In an attempt to evaluate possible proliferation of Sp in the uremic intestine, we also enumerated the population of alkalophilic bacteria in the feces obtained from both placebo and Sp-fed animals. No significant differences in the counts of alkalophilic bacteria were observed after 8 weeks of daily feeding with 10^9 cfu of Sp (log cfu/g 9.50 ± 0.26) as compared with baseline (log cfu/g 9.30 ± 0.39), suggesting that Sp does not proliferate in the intestine.

Discussion

Current therapies to remove uremic solutes for the ESRD patient include peritoneal dialysis, hemodialysis, and kidney transplantation. Each of these costly and time-consuming regimens is associated with high patient morbidity. These types of treatments are predominantly found within industrialized (wealthy) countries, and therefore patients of developed countries are more likely to have extended life expectancy. In underdeveloped countries, uremia is generally untreated and patients seem to have a lower life expectancy. Predialysis patients may be treated with protein and potassium diet restrictions, erythropoietin, and to some extent, compound specific interventions such as Renagel or Kayexalate, but no generally applicable therapies to slow the progression of kidney disease are available.

In the recent years, efforts have been undertaken to mitigate uremia in animals and humans by administration of live cultures of naturally existing[5] or genetically engineered microbes.[6,38] Taken together, these studies not only prove the concept of "enteric dialysis," but also give some promise to the millions of people with kidney dysfunction of different etiology.

In the present study, we summarized a few years of work focused on investigation of the potential use of Sp as a urea-targeted component in bacteriotherapy of uremia. A number of questions were addressed in the present study: (1) ability of Sp to survive and hydrolyze urea in conditions simulating gastrointestinal tract; (2) effects of Sp feeding on gut microbial ecosystem and fermentation parameters in the colon; and (3) ability of Sp to produce the desired effects *in vivo*.

In a series of *in vitro* experiments, we proved that both vegetative cells and spores can survive exposure to gastric juice and thus can be delivered to the intestine upon ingestion.

Although we cannot exclude the possibility that this bacterium will proliferate in the uremic intestine, our *in vitro* data suggest that it is not likely, most probably due to the inappropriate pH. Enumeration of alkalophilic species in the feces of 5/6th nephrectomized rats also did not indicate active proliferation of Sp in the rat intestine, even though Sp retained the ability to hydrolyze urea both *in vitro* and *in vivo*.

Our data also indicate that rates of urea removal are determined by urea concentration, pH, and, to some extent, by nutrient availability (**Figure 3**). As a result, amount of urea, which can be removed by 10^9 cfu of bacteria delivered to the intestine, is expected to vary between 21 ± 4.7 (measured at pH 5.0 and 100 mg/dl urea, concentration about 50% higher than normal physiological levels), and 228 ± 6.7 (measured at pH 7.5 and 300 mg/dl, advanced uremia). Simple calculations based on the volume of blood in human body and difference between uremic and normal levels of urea allows estimating that about 20–25 g of urea per day must be removed. Our data suggest that 10^9 cfu of Sp in freeze-dried form, although not likely to proliferate in the gastrointestinal tract, can remove up to 1.125 g urea in 5 hours. According to these data, the targeted removal of about 20–25 g urea per day (in advanced uremia) will require the daily ingestion of approximately 10^{10}–10^{11} viable cfu. Our data also suggest that Sp will remove urea from the uremic matrix in an amount that will prove beneficial to persons suffering from any degree of renal insufficiency. Importantly, because the rate of hydrolysis is proportionate to the BUN levels, the same anticipated regimen can be used by all categories of patients.

Although Sp is occasionally isolated from human feces and no pathologic reactions have been associated with this bacterium, it is hard to exclude the possibility that ingested Sp may cause infections in some patients with compromised immune system. From that perspective, every bacterial strain proposed for human or animal consumption needs to be tested for susceptibility to common antibiotics. Consumption of live bacteria may also be hazardous if ingested bacteria contain plasmids with antibiotic resistance genes that can be horizontally transferred to other bacterial species, including pathogenic bacteria, in the intestine. Although no criteria for the determination of antimicrobial susceptibilities exist for Sp, we were able to demonstrate inhibition of growth of organism to several classes of antibiotic using Kirby-Bauer method (data not shown). Further analysis is required to address this issue.

Theoretically, 57% of urea mass is transformed into NH_3 with the remaining 43% released as CO_2. The primary concern was: What happens to the theoretically large amount of ammonia produced by the hydrolysis of urea in the intestine? Our *in vitro* results indicate that live cultures of Sp liberate far less ammonia than theoretically expected (**Figure 2**). In a growing culture, this can be attributed to the uptake of ammonia as a source of nitrogen. Alternatively, ammonium can be sequestered inside the cell for alkalinization of the cytoplasm.[30] As demonstrated by the SHIME study, in normal physiological conditions, only about 10% increase in ammonia production in colon *transversum* and colon *descendans* is observed as a result of Sp feeding. Considering that most of the fermentation occurs in colon *ascendans,* these changes should not have dramatic effects on microbial community. This conclusion was also supported by enumeration of marker organisms in the SHIME (**Table 2**), which suggests that supplementation with Sp does not cause disturbances in the intestinal microbial ecosystem. Although changes in SHIME *Lactobacilli* population were significant, they cannot be considered dramatic since variations of a similar range are normally observed in healthy individuals.[39] Importantly, no effects of Sp on lactic acid bacteria counts were registered *in vivo* in Sp-fed uremic rats. In addition, positive effects of Sp on fermentation parameters and total aerobes and *Clostridia* counts in the SHIME may suggest beneficial impact of Sp overall.

Another key concern was that CO_2 produced by ureolysis may cause bloating and flatulence. Although these or other adverse reactions were not registered in either nonnephrectomized rats fed Sp (10^8 cfu/day for 16 weeks, n = 12, separate study, data not shown) or 5/6th nephrectomized rats treated with Sp in the present study (10^9cfu/day), we cannot exclude the possibility that such reactions may occur in Sp-treated patients. Because bloating emerges as a minor adverse reaction, patients may be willing to endure the discomfort in exchange for freedom from dialysis.

Taken together, our *in vitro* studies suggest that Sp may be a suitable candidate to mitigate urea accumulation *in vivo* without inducing a significant increase in free ammonia, disturbing intestinal microbiota, or causing severe adverse reactions. This conclusion is supported by *in vivo* studies, which demonstrated beneficial effects of Sp feeding in an animal model of CKD. Supplementation of 10^9 cfu throughout the study attenuated BUN levels in 5/6th nephrectomized rats, slowed the progression of azotemia observed in placebo-fed animals, and prolonged the lifespan of uremic animals. Human trials are required to prove that Sp can be used as a biopharmaceutical.

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