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Inhibition of urinary stone disease by a multi-species bacterial network ensures healthy oxalate homeostasis

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Abstract

The incidence of urinary stone disease is rapidly increasing, with oxalate being a primary constituent of approximately 80% of all kidney stones. Despite the high dietary exposure to oxalate by many individuals and its potential nephrotoxicity, mammals do not produce enzymes to metabolize this compound, instead relying in part on bacteria within the gut to reduce oxalate absorption and urinary excretion. While considerable research has focused on isolated species of oxalate-degrading bacteria, particularly those with an absolute requirement for oxalate, recent studies have pointed to broader roles for microbiota both in oxalate metabolism and inhibition of urinary stone disease. Here we examined gut microbiota from patients with and live-in individuals without urinary stone disease to determine if healthy individuals harbored a more extensive microbial network associated with oxalate metabolism. We found a gender-specific association between the gut microbiota composition and urinary stone disease. Bacteria enriched in healthy individuals largely overlapped with those that exhibited a significant, positive correlation with *Oxalobacter formigenes*, a species presumed to be at the center of an oxalate-metabolizing microbial network. Furthermore, differential abundance analyses identified multiple taxa known to also be stimulated by oxalate in rodent models. Interestingly, the presence of these taxa distinguished patients from healthy individuals better than either the relative abundance or colonization of *O. formigenes*. Thus, our work shows that bacteria stimulated by the presence of oxalate in rodents may, in addition to obligate oxalate users, play a role in the inhibition of urinary stone disease in man.

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Availability of data

Sequence reads are available at the Sequence Read Archive under accession #SRP140933.

DISCLOSURE

All the authors declared no competing interests.

Keywords

intestinal microbiome; oxalate; *Oxalobacter formigenes*; urinary stone disease; urolithiasis

Hyperoxaluria is defined as an excessive amount of oxalate in the urine, usually >45 mg/d, and is a major contributor to calcium oxalate kidney stone disease.¹ Oxalate is endogenously produced in the liver as part of normal metabolism and is also absorbed in the intestine from oxalate-containing foods,^{2–7} especially when unopposed by dietary constituents, such as calcium, that form an insoluble complex with oxalate in the gastrointestinal tract. A direct link between intestinal bacteria and calcium oxalate kidney stone disease came after the discovery of *Oxalobacter formigenes*. This commensal intestinal bacterial species uses oxalate as its primary nutrient source because of the expression of a specialized set of enzymes capable of rapidly degrading high concentrations of the compound and prevent its absorption into circulation.^{8,9} A mechanism by which *O. formigenes* is able to stimulate oxalate secretion from circulation back into the gut has also been suggested.¹⁰ However, numerous studies have investigated the colonization status of recurrent kidney stone formers and non-stone-forming controls and shown that the absence of *O. formigenes* alone is not causative of stone disease, as some recurrent stone formers are colonized whereas some nonstone formers are not. In fact, studies report colonization rates for patients with urinary stone disease (USD) by *O. formigenes* ranging from 8% to 100% and for healthy individuals from 11% to 100%, with some studies reporting no significant differences.^{11–30} Furthermore, the presence of *O. formigenes* is associated with significantly lower urinary oxalate in only 55% of the studies that reported these results. Finally, in a recent clinical trial involving patients with primary hyperoxaluria, *O. formigenes* given as a probiotic daily for 8 weeks failed to reduce urinary oxalate excretion, despite substantial recovery of *O. formigenes* DNA from stool samples.³¹ These results suggest that bacterial species in addition to *O. formigenes* may be important to convey protection against USD.

Recent studies have gone beyond *O. formigenes* and investigated differences at the genus level in the intestinal microbiota of patients with a history of stones versus non-stone-forming controls.^{26,32–34} Consistent among these studies, the *Bacteroides* genus were enriched in patients with USD whereas *Prevotella* was enriched in healthy individuals. Furthermore, in patients with 24-hour urine results, the presence of *Eubacterium* species was inversely correlated with urine oxalate.³² In 3 studies, there was no significant difference in *Oxalobacter* between healthy and USD groups. However, in 1 study, when *Oxalobacter-specific* primers were used, 100% of healthy individuals were colonized by *O. formigenes* compared with only 17% of patients with USD.¹⁴ In that same study, similar to herbivorous rodents consuming high amounts of oxalate, patients were found to harbor a diversity of other oxalate-degrading bacteria, as assessed by the presence of the *frs* gene.^{26,35} Finally, a recent study that performed a comparative shotgun metagenomic analysis found that patients with USD harbored fewer and less diverse oxalate-degrading genes compared with controls, even though there was no significant difference in *Oxalobacter*.³⁴ Although these studies did show clear differences in the gut microbiota of patients with USD versus healthy controls and that patients with USD harbored lower diversity and abundance of oxalate-degrading

bacteria, they did not identify cooperative networks of bacteria that may be responsible for oxalate metabolism and/or inhibition of USD.

To better understand microbial networks important to maintain overall oxalate homeostasis, Miller *et al.* studied the intestinal microbiome of *Neotoma albigula* (white-throated wood rat), a mammalian herbivore whose foregut contains a microbiome highly efficient at degrading dietary oxalate, even at concentrations of 12%.³⁶ Constituents of the wood rat's microbiome include *Oxalobacter*, *Lactobacillus*, *Clostridium*, and *Enterococcus* among other potential oxalate-degrading bacteria.^{35–39} These studies identified a response by the intestinal microbiome to increasing dietary oxalate that stimulated a consistent core community of microbes, centered on the Oxalobacteraceae, which appears essential for oxalate degradation. To determine whether a similar oxalate-degrading microbiome plays a role in maintaining overall oxalate homeostasis in non-stone-forming humans and whether they play a role in stone disease in general, we examined differences in the gut microbiota of patients with USD versus cohabitating healthy controls, with emphasis on the microbial network associated with *Oxalobacter*, as currently this is the only bacterial species linked to stone disease. Furthermore, species symbiotically linked to *O. formigenes* (hence part of an *Oxalobacter* network) but without a direct role in oxalate breakdown could play a role in the formation of nonoxalate stone types. This provides insight into not only a potential role for an oxalate-degrading microbial network in calcium oxalate USD but also a potential role for associated dysbiosis in patients who had nonoxalate USD.

RESULTS

In the present study, we recruited 17 patients with USD along with 17 live-in controls without USD. No participant had antibiotic exposure within at least 1 month before sample collection, and none reported having undergone medical procedures and/or supplementation, resulting in a significantly altered intestinal microbiome composition. Of all patients, 10 were calcium oxalate stone formers confirmed either by stone analysis or by computed tomography scan (Hounsfield unit measurement), 2 patients had uric acid stones, 2 patients had cystine stones, 1 patient had a struvite stone, and 2 patients had a stone of unknown type. The patient group consisted of 12 men and 5 women, with a mean age of 58.0 ± 12.1 years and a mean body mass index of 30.52 ± 5.42 kg/m², and all but 1 had a significant history of stone recurrence. The control group consisted of 5 men and 12 women, with a mean age of 59.18 ± 10.73 years and a mean body mass index of 25.85 ± 5.87 kg/m² (Table 1). Habitual dietary intake was collected from all patients and controls to determine a potential effect of varying diets on the micro-biome composition. Stool samples were collected from all participants for high-throughput sequencing of the 16S ribosomal RNA (rRNA) gene.

There were no differences between patients with USD and controls with regard to intake of nutrients and other dietary parameters (Table 2), including macronutrient composition and estimations of potential renal acid load of diet and net endogenous acid production. When stratified by sex, there were no differences between male patients and controls, nor between female patients and controls, with regard to any of the dietary parameters assessed. However, there were differences between men and women within the patient group, specifically men

had higher potential renal acid load of diet and net endogenous acid production than did women (-0.67 vs. -12.68 ; $P=0.043$ from 2-tailed t -test and 42.5 mEq/d vs. 32.5 mEq/d; $P=0.013$ from 2-tailed t -test, respectively).

The gut microbiota of patients with USD and healthy controls were characterized through sequencing. Sequencing of the V4 region of the 16S rRNA gene yielded a total of 2,207,898 high-quality sequences from all 34 individuals with a total of 7964 unique operational taxonomic units (OTUs) defined at the 97% similarity level. More than 99% of OTUs were classified to the level of phylum, with 61% classified to the genus level. Both patients and controls were dominated by the Firmicutes phylum (~ 52% of the total for both), followed by Bacteroidetes (22% for both). Taxonomic analysis revealed a significant reduction in the Tenericutes phylum present within the gut microbiota of patients versus controls (Figure 1a). In addition, although there were no significant differences between groups with regard to alpha diversity (Table 3), there was a sex-specific difference in gut microbiota composition (beta diversity) between patients and controls when the relative abundance of OTUs was taken into consideration (weighted UniFrac analysis) (Figure 1b and c).

Differential abundance analysis revealed that 103 OTUs were significantly enriched in healthy individuals, with only 62 enriched in patients (Figure 2a; Supplementary Table S1). Although there was a trend toward higher relative abundance and colonization of *O. formigenes* in healthy individuals, the difference was not significant between groups (Figure 2b and c). A total of 149 OTUs exhibited a significant positive correlation with *Oxalobacter* sp. across the entire data set (Supplementary Table S2). In terms of bacteria that correlated with *Oxalobacter*, there was an order of magnitude higher number of co-occurrence interactions in the healthy group than in the USD group (Figure. 2d).

To further determine whether healthy individuals harbored a more robust microbial network associated with oxalate metabolism than did patients with USD, we compared the list of bacteria enriched in the healthy and USD groups from this study with the list of bacteria exhibiting a significant positive correlation with *Oxalobacter* from *N. albigula* that were stimulated by oxalate (Figure 3).³⁶ Some taxa, such as *Ruminococcus* (from both the Ruminococcaceae and Lachnospiraceae families) and *Oscillospira*, had OTUs that were consistently present in healthy individuals and not in patients, were associated with the presence of *Oxalobacter*, and were stimulated by oxalate in the rodent model. However, other taxa, such as *Bacteroides*, *Akkermansia*, *Bifidobacterium*, *Coprococcus*, and *Odoribacter*, had OTUs in all 4 groups.

DISCUSSION

In the present study, we sought to address important gaps in knowledge regarding the role of the intestinal microbiome beyond *O. formigenes* and other oxalate-degrading bacteria in recurrent USD. We opted to take a global approach to the analysis rather than focus on specific subsets of patients such as those with hyperoxaluria, as we wanted to identify potential differences that exist across individuals who had USD. In the present study, we focused our analysis around microbial networks associated with oxalate metabolism, as this to date has been identified as the only metabolic pathway associated with recurrent kidney

stone disease. Overall, we hypothesized that healthy individuals would harbor a more robust microbial network associated with oxalate metabolism than would patients with USD. Previous work shows that oxalate metabolism is associated with a diverse community of bacteria rather than a single species, indicative of metabolic cooperation that may help to persistently maintain oxalate-degrading bacteria and their function in a complex and competitive environment.^{36,37} Network analysis of bacteria associated with *O. formigenes* is one way to identify metabolically cooperative bacteria using only a snapshot of the microbiota, which is important for clinical studies.

Overall, we found a significant reduction in the Tenericutes phylum in all patients with USD compared with the healthy population (Figure 1a). In addition, there was a sex-specific significant difference in community structure but not in membership between the microbiota of patients and controls (Figure 1b and c), which may reflect the sex-based differences in stone risk.⁴⁰ When looking at the differential abundance of specific OTUs within each of the groups, we found 62 OTUs to be enriched in patients versus 103 OTUs enriched in non-stone-forming controls. Interestingly, *O. formigenes* was conspicuously absent from the list of OTUs enriched in controls, suggesting that other bacterial species are more important to prevent USD and/or oxalate homeostasis. In addition, we found that the number of co-occurrence interactions with bacteria associated with *Oxalobacter* discriminated patients from healthy controls more effectively than looking at the presence or absence of *O. formigenes* alone.

To identify bacterial taxa with importance in oxalate homeostasis, we compared the list of bacteria enriched in healthy individuals and associated with *Oxalobacter* sp. (from this study) with the list of bacterial species stimulated by dietary oxalate in *N. albigula*.³⁸ This herbivorous rodent is ideal to study microbial oxalate metabolism, as the species has consumed a high oxalate diet in the wild for thousands of years and thus has not lost any bacteria because of laboratory rearing, drastic dietary changes, or antibiotic exposure, as is the case for laboratory rodents and humans.³⁵ Our comparison revealed 2 important points. First, some taxa, such as *Ruminococcus* (from both the Ruminococcaceae and Lachnospiraceae families) and *Oscillospira*, had OTUs that (i) were consistently present in healthy individuals and not in patients, (ii) were associated with the presence of *Oxalobacter*, and (iii) were stimulated by oxalate in rodents. This corroborates previous studies that examined the differences in microbiota composition between USD and healthy populations,^{26,33} which found that OTUs involving *Ruminococcus* and *Oscillospira* were enriched in controls. Stern *et al.*³² did not make these observations, but reported differences at the genus level rather than OTU-level differences. The second point revealed by our comparison is that some taxa, such as *Bacteroides*, *Akkermansia*, *Bifidobacterium*, *Coprococcus*, and *Odoribacter*, had OTUs stimulated in all compared groups, a finding that largely concurred with both the Suryavanshi and Stern studies.^{26,32} Thus, our data provide preliminary evidence that taxa previously associated with oxalate metabolism in rodents also convey a protective role against USD in humans. Adding weight to this is our finding that OTUs that were different between the patient and control intestinal microbiota included taxa, such as *Desulfovibrio* and *Methanobrevibacter*, which engage in sulfate reduction, methanogenesis, and acetogenesis. These bacterial species likely associate closely with *O. formigenes* as methanogens, acetogens, and sulfate-reducing bacteria use formate, the major by-product of

oxalate metabolism by *O. formigenes*, which is why we think they co-associate.^{41,42} Similarly, acetogens use CO₂, another major by-product from oxalate breakdown, to produce acetate, a beneficial nutrient for the host and other microbes.⁴³ The fact that *O. formigenes* does not express enzymes necessary to assimilate formate or CO₂ increases the likelihood that it has to rely on other bacterial species for these functions. This underscores the likelihood that bacteria other than those directly involved in oxalate breakdown play a significant role in oxalate metabolism *in vivo*, as suggested previously.³⁷ This observation may further explain why recolonization with *O. formigenes* alone has only transient results.²³ Future microbiological studies aimed at understanding the fine interplay between members of the oxalate microbiome at the functional level will provide a better insight into the role for each member and their importance in maintaining overall non-stone-forming environment.

An interesting finding was the fact that disturbances in the oxalate-degrading microbial network were also present in patients with nonoxalate stones. In total, at least 5 of the patients included in this study who had stones that were not calcium oxalate based, yet their microbiome showed dysbiosis that included members of the oxalate-degrading microbial network. Although the sample number may be low, the data do suggest that disturbances in the intestinal microbiome, including members associated with the oxalate-degrading microbial network identified here, may increase the risk of developing other stone types and warrants further investigation in a larger study.

The absence of dietary differences between cases and controls suggests that the observed differences in gut microbiota were related to the pathophysiology of stones and/ or other factors and not to diet. Although nutrient analyses of the results of the Canadian version of the National Institutes of Health Diet History Questionnaire (C-DHQ-I) did not include oxalate (because of limitations of nutrient analysis software), surrogate measures of oxalate intake, including whole grains, vegetables (including dark green leafy vegetables as a separate category), potatoes, nuts and seeds, and soy, revealed no differences. In addition, dietary factors that influence the absorption of dietary oxalate from the gastrointestinal tract (e.g., calcium and magnesium) were not different between groups, further supporting a role for differences in intestinal microbiome composition in affecting the absorption of components relevant to stone formation.

The intestinal microbiome is known to play a significant role in maintaining overall health, and its dysbiosis has been linked to numerous disease states including USD. For calcium oxalate USD specifically, the primary species of interest has been *O. formigenes*, although other facultative oxalate-degrading species have been the subject of research as well.^{8,13,23,31,44–50} Several factors suggest that although *O. formigenes* and other oxalate-degrading bacteria are indeed associated with oxalate metabolism in the gut, they are not solely responsible for the function, nor are they sufficient to inhibit calcium oxalate USD. First, although a majority of patients are found not to be colonized by this strict oxalate-degrading bacterium, the same is true of non-stone-forming individuals. In addition, many patients with calcium oxalate USD are colonized by *O. formigenes*.⁵¹ Second, although probiotics containing *O. formigenes* or a mix of facultative oxalate-degrading bacteria do reduce urinary oxalate excretion, their effect, if at all, is typically ephemeral.

13,23,31,44,47–50,52 In contrast, given whole fecal transplants from *N. albigula*, mammals whose gastrointestinal tracts harbor highly efficient bacterial oxalate-degrading networks, Sprague-Dawley rats exhibit a marked and persistent decrease in urinary oxalate.^{37,38} Third, the intestinal microbiome is a highly symbiotic environment consisting of complex and integrated functional microbial networks.^{53,54}

In conclusion, our results suggest that healthy oxalate homeostasis in the gastrointestinal tract may not be attributed to the action of *O. formigenes* alone, but may rather involve a collaborative effort between numerous bacterial species, including *Ruminococcus* and *Oscillospira*. This would certainly be consistent with the highly symbiotic environment of the intestinal microbiome, an example of which is the co-colonization in our samples of methanogens species with *O. formigenes*. Furthermore, our data suggest a potential role for the lost members of the oxalate microbiome in increasing the risk of forming nonoxalate stones, which is consistent with the highly symbiotic nature of the intestinal microbiome and requires further investigation. Although the results of the present study were based on a single snapshot of the gut microbiota, they provide a strong impetus for broader studies of the role of the oxalate-degrading microbial network and USD.

METHODS

The study was approved by the Clinical Research Ethics Board of the University of British Columbia, and informed consent was obtained from all participants. All included subjects had no antibiotic exposure within 30 days before providing the sample, and based on detailed medical history obtained at the time of enrollment, they had no interventions/treatments that affected the intestinal microbiome composition. However, we did not determine the number of daily bowel movements per patient, which could affect the microbiome composition downstream. Patients had at least 1 recurrence of their stones and no family history of primary hyperoxaluria, whereas controls live in the same household as patients and had no personal or family history of USD. Subject demographic characteristics are given in Table 1.

Power analysis based on the results of previous studies of the microbiota composition revealed that our sample size gave a 65% power of detecting a difference in community composition and differential abundance.³⁷

Dietary intake of both cases and controls was assessed using the C-DHQ-I. This instrument is a publicly available food frequency questionnaire consisting of 134 food items—based on the national dietary data from the National Health and Nutrition Examination Surveys from 2001 to 2002, from 2003 to 2004, and from 2005 to 2006—and 11 dietary supplement questions. The C-DHQ-I queries subjects about intake in the past year, assessing for habitual intake, and includes questions about portion size. The paper-and-pencil version of the questionnaire was provided to each subject before his/her fecal sample was collected. Data from the C-DHQ-I were analyzed using the Diet*Calc software developed by the National Cancer Institute specifically for this instrument. Nutrient and food group intake estimates were calculated.

Fecal samples were collected by study participants into provided containers on the morning of sample delivery. Upon arrival at our facility (within 4 hours of defecation), fecal samples were stored at 4° C before being aliquoted into microfuge tubes and subsequent storage at – 80° C until DNA extraction.

Fecal DNA was extracted and purified using the QIAamp DNA Stool Mini Kit (51504, Qiagen, Hilden, Germany) according to the manufacturer's instructions with modifications to cell lysis buffer (4% [w/v] sodium dodecylsulfate, 500 mM NaCl, 50 mM ethylenediamine tetraacetic acid, 50 mM tris(hydroxymethyl)-aminomethane, pH 8.0) and use of additional glass lysis beads (0.3 g of 0.1 mm beads and 0.1 g of 0.5 mm beads) for a more thorough lysis of Firmicutes bacteria.

A 16S rRNA library was prepared from the fecal DNA on the basis of the protocol by Kozich *et al.*⁵⁵ Extracted DNA was amplified using the Phusion Hot Start II DNA Polymerase (2 U/ml) kit (F549S, Thermo Fisher Scientific, Waltham, MA) in 50 µl reactions according to the manufacturer's instructions, with the following modifications to the polymerase chain reaction (PCR) cycle: initial denaturation at 98 °C for 2 minutes, 30 cycles of 98 °C for 20-second extensions, 55 °C for 15-second extensions, and 72 °C for 30-second extensions, followed by a final extension at 72 °C for 10 minutes and holding at 4 °C. To validate PCR success, a random subset of PCR products was analyzed for visible bands on gel electrophoresis. The PCR products were cleaned using Agencourt AMPure XP beads (A63880, Beckman Coulter, Brea, CA) with a bead to sample ratio of 0.8:1. The cleaned PCR products were normalized using the SequalPrep Normalization Plate Kit (A1051001, Invitrogen, Carlsbad, CA) to a concentration of 1 to 2 ng/µl. Five microliters of each normalized sample were pooled into a single library and further concentrated using the DNA Clean & Concentrator-5 Kit (D4013, Zymo Research, Irvine, CA). The pooled library was analyzed on the Agilent Bioanalyzer using the High-Sensitivity dsDNA assay (5067–4626, Agilent, Santa Clara, CA) to determine the approximate library fragment size and to verify library integrity. The QIAquick Gel Extraction Kit (28704, Qiagen) was used to extract properly sequenced 16S rRNA amplicons in the pooled library and exclude unintended amplicons. The concentration of the final pooled library was determined using the KAPA Library Quantification Kit for Illumina (KK4824, Kapa Biosystems, Wilmington, MA). The library was then diluted to 4 nM and denatured into single strands using 0.2 N NaOH. The final library loading concentration was 8 pM, with an additional 20% PhiX (FC-110–3001, Illumina, San Diego, CA) spike-in for sequencing quality control. The 16S rRNA pooled library was then sequenced on an Illumina MiSeq platform.

Unless otherwise noted, all analyses were performed using QIIME.⁵⁶ Raw sequencing data were demultiplexed with default parameters, and OTUs were assigned *de novo* at a 97% homology cutoff by using UCLUST.⁵⁷ Data were filtered to remove mitochondria, chloroplasts, and sequences with <10 representations across the entire data set. Filtered data were used to summarize taxa, determine which OTUs differed between groups, and quantify bacteria that co-occur with *Oxalobacter* sp. (discussed below).

Before comparative analyses, data were normalized with the DESeq2 algorithm, which executes a negative binomial Wald test while maintaining rare taxa.^{58,59} For alpha diversity,

Margalef species richness index, evenness, the Shannon index, and phylogenetic diversity were calculated. In addition, beta diversity was calculated using both unweighted (membership) and weighted (structure) UniFrac analyses, followed by a *post hoc* 2-way permutational multivariate analysis of variance against USD status and sex as factors.⁶⁰ The differential abundance of OTUs between USD and healthy groups was calculated using a Wald test, which determines significance by the log₂ fold change of the normalized OTU abundance divided by its SE. The resulting *P* values were adjusted to account for false discoveries.⁵⁸

To compare differences between groups in the microbial network associated with oxalate metabolism, several metrics were calculated. First, the normalized relative abundance of *Oxalobacter* sp. was compared using the *t*-test. Second, the proportion of individuals colonized by *Oxalobacter* sp. in each group was compared using relative risk analysis in R statistical software (R Development Core Team, Auckland, New Zealand). Finally, with the assumption that *Oxalobacter* is a central component of a broader microbial network associated with oxalate metabolism, we quantified the bacteria that exhibited a relative abundance that significantly and positively correlated with *Oxalobacter* as assessed by false discovery rate-corrected Pearson correlations. The resulting list was used to quantify the co-occurrence network in the healthy and USD groups. Co-occurrence was determined using the SparCC algorithm, by group, as previously described.^{36,61}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1 |. Characterization of the whole gut microbiota between healthy and urinary stone disease (USD) cohorts.

(a) Phylum-level profile of the microbiota. Statistical analysis (t -test) reveals a significant reduction in the Tenericutes phylum in patients with USD ($P = 0.012$). (b) Beta diversity of the microbial community membership, based on an unweighted UniFrac analysis. Circles represent the multivariate homogeneity of dispersion around a centroid for each group comparison. (c) Beta diversity of the microbial community structure, based on weighted UniFrac analysis. Circles represent the multivariate homogeneity of dispersion around a centroid for each group comparison. Twoway permutational multivariate analysis of variance reveals a significant sex*USD status response for weighted (c) but not unweighted (b) UniFrac analysis (weighted UniFrac - USD status: $P = 0.071$; sex: $P = 0.221$; sex*USD status: $P = 0.018$; unweighted UniFrac - USD status: $P = 0.181$; sex: $P = 0.335$; sex*USD status: $P = 0.589$). NF, no female; NM, no male; PCoA, principle coordinates analysis; YF, yes female; YM, yes male.

Figure 2 |. Differential abundance analysis of functionally relevant bacteria between urinary stone disease (USD) and healthy cohorts.

(a) Total number of differentially abundant operational taxonomic units (OTUs) between groups. Red dots indicate significantly enriched OTUs (103 enriched in the healthy cohort; 62 enriched in the USD cohort). (b) Colonization by *Oxalobacter* for each group (relative risk analysis, $P = 0.06$). (c) Relative abundance of *Oxalobacter* for each group (t -test, $P = 0.06$). (d) Number of co-occurrence interactions of bacteria that exhibit a significant positive correlation to *Oxalobacter* in each group.

Figure 3 |. Quantification of the oxalate microbiome. Genera enriched in healthy individuals or positively correlated to *Oxalobacter* sp. were compared with those genera stimulated by oxalate in *Neotoma albigula*. Blue indicates genera significantly enriched in the urinary stone disease (USD) or healthy groups, correlated to *O. formigenes*, or stimulated by oxalate, and violet indicates nonsignificant associations.

Table 1|**Patient metadata**

Metric	Controls	Patients with stone
Male sex	29	71
Female sex	71	29
Age (yr)	59.19 ± 10.73	58.0 ± 12.16
BMI (kg/m ²)	25.85 ± 5.87	30.52 ± 5.42
Obese	18	42
Nonobese	71	47
Calcium oxalate	NA	53
Cystine	NA	12
Uric acid	NA	12
Struvite	NA	6
USD history (yr)	NA	2–30

BMI, body mass index; NA, not applicable; USD, urinary stone disease. Data are mean ± SD or percentage.

Table 2|

Data reflect dietary intake analysis from food frequency questionnaires for both cases and controls

	Cases	Controls	P-value
Energy (kcal/d)	2085 (1859)	1922 (1437)	0.65
PRAL ^a	-5.2 (-6.0)	-4.0 (0.0)	0.82
NEAP ^b (mEq/d)	39 (39)	40 (40)	0.79
Protein (g/d)	87 (81)	79 (65)	0.60
Fat (g/d)	84 (79)	75 (55)	0.52
Carbohydrates (g/d)	249 (226)	238 (182)	0.81
Saturated fat (g/d)	26 (26)	23 (17)	0.58
MUFA ^c (g/d)	33 (32)	29 (23)	0.47
PUFA ^d (g/d)	18 (18)	16 (13)	0.50
Added sugar (teaspoons/d)	13 (10)	13 (6.0)	0.82
Fiber (g/d)	22 (21)	20 (20)	0.66
Insoluble fiber (g/d)	15 (14)	14 (13)	0.46
Soluble fiber (g/d)	7.7 (7.3)	7.3 (7.2)	0.75
Fructose (g/d)	23 (18)	22 (18)	0.85
Whole grains (servings/d)	1.4 (1.2)	0.92 (0.65)	0.17
Refined grains (servings/d)	4.5 (4.1)	4.6 (2.8)	0.94
Vegetables (servings/d)	4.7 (4.5)	5.3 (4.9)	0.57
Dark green leafy vegetables (servings/d)	0.80 (0.56)	0.98 (0.50)	0.59
Deep yellow vegetables (servings/d)	0.43 (0.31)	0.51 (0.47)	0.56
Dry beans/peas (servings/d)	0.12 (0.09)	0.12 (0.09)	0.93
White potatoes (servings/d)	0.65 (0.44)	0.48 (0.30)	0.44
Other starchy vegetables (servings/d)	0.26 (0.23)	0.29 (0.22)	0.63
Tomatoes (servings/d)	0.59 (0.42)	0.59 (0.43)	0.99
Other vegetables (servings/d)	1.8 (1.2)	2.3 (1.9)	0.45
Fruits (servings/d)	3.1 (2.1)	2.8 (2.3)	0.71
Citrus, melon, and berries (servings/d)	1.4 (0.77)	0.97 (0.80)	0.31
Other fruits (servings/d)	1.8 (1.4)	1.8 (1.5)	0.87
Dairy (servings/d)	1.5 (1.3)	1.5 (1.2)	0.86
Dairy milk (servings/d)	0.80 (0.45)	0.56 (0.44)	0.35
Yogurt (servings/d)	0.29 (0.31)	0.38 (0.26)	0.54
Cheese (servings/d)	0.44 (0.34)	0.52 (0.27)	0.65
Meats ^e (oz/d)	4.5 (4.0)	4.0 (3.2)	0.58
Fish (oz/d)	0.48 (0.46)	0.63 (0.50)	0.34
Nuts and seeds (oz/d)	0.56 (0.41)	0.31 (0.30)	0.11
Alcohol (drinks/d)	0.37 (0.11)	0.26 (0.15)	0.50
Vitamin C (mg/d)	135 (92)	119 (125)	0.56

	Cases	Controls	P-value
Vitamin B ₆ (mg/d)	1.9 (1.8)	1.9 (1.7)	0.81
Calcium (mg/d)	921 (769)	852 (846)	0.66
Magnesium (mg/d)	395 (415)	343 (323)	0.30
Sodium (mg/d)	3298 (3309)	3090 (2567)	0.73
Potassium (mg/d)	3691 (3607)	3358 (3200)	0.45

Data are means and medians per group. *P*-values are from 2-sided t-tests.

^aPRAL, potential renal acid load of foods (validated calculation involving intake of protein, phosphorus, potassium, magnesium, and calcium).

^bNEAP, net endogenous acid production (validated calculation involving intake of protein and potassium).

^cMUFA, monounsaturated fatty acid.

^dPUFA, polyunsaturated fatty acid.

^eIncludes flesh from all mammals, fowl, fish, and seafood.

Table 3|

Metrics for alpha diversity

Variable	Margalef species richness index	Evenness	Shannon index	Phylogenetic diversity
Mean (urinary stone disease)	32.41	0.67	5.35	55.50
SE	5.61	0.03	0.26	8.77
Mean (healthy)	37.95	0.72	5.94	64.53
SE	5.73	0.02	0.17	8.11
<i>P</i> (<i>t</i> -test)	0.49	0.15	0.07	0.46

Means were compared using a *t*-test.