Characterizing the Role of the Microbiome in Kidney Stone Disease

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Abstract

The goal of this thesis was to increase understanding of the role of the human microbiome in kidney stone disease, from stone nidus formation through to surgical stone treatment, using a combination of *in vitro*, *in vivo*, and human clinical investigations.

We first optimized our methods of sample collection for the use of such protocols in clinical studies involving urinary and gut microbiota investigations. We developed a novel method of fecal sampling that is amenable to study participants, inexpensive, and results in reliable downstream sequencing results.

We then utilized this sampling methodology in clinical investigations into the microbiota of surgical kidney stone patients using a systems-level approach. We determined that there is a microbiota present in all kidney stone crystalline compositions, which was previously unknown. The urinary microbiome was distinct between stone formers and controls in both microbiota composition and based on targeted metabolomics. Stone formers had higher urinary oxalate concentrations and elevated relative abundance of inflammatory and uropathogenic microbes throughout the course of stone treatment. In the gut, stone formers had altered microbial community composition at both a taxonomic and functional level, with implications for uropathogen abundance and host oxalate homeostasis. We determined that the gut has a significant and multipronged contribution to kidney stone formation.

In a cohort of primarily nephrolithiasis-related urological patients, we further characterized the microbiota associated with ureteral stents, an almost ubiquitous component of surgical stone treatment. We determined that the stent microbiota is reproducible and patient specific, and not represented by the urinary microbiota. Patient factors and comorbidities drive the stent microbiota composition, and neither the microbial community nor degree of stent encrustation were altered by antibiotic use, indicating that perhaps antibiotic use in stent patients needs recalibration.

Finally, we investigated host-microbe interactions in stone formation using *in vitro* and *in vivo* models, specifically how uropathogens may contribute to stone formation and how probiotics may provide therapeutic benefit. We determined that both pathogenic and
beneficial bacteria have the capability of shaping stone disease progression and should be considered in stone treatment.

Collectively, these studies have shed light on the contribution of microbes in this prevalent and morbid condition, and elucidated novel ways to harness the microbiome in nephrolithiasis management.

**Keywords**

Kidney stone disease, microbiome, next generation sequencing, endourology, *Drosophila melanogaster*, ureteral stents
Summary for Lay Audience

Kidney stone disease affects approximately 10% of the population; it has been described as a pain worse than childbirth and its treatment is a financial drain to the health care system. Many people develop stones time and time again, but why this happens is not well known. This project aimed to determine if the microbiota, the bacteria that live within us, is a factor in this disease. We determined that the bacteria, and the products they make, in the gut and urinary tract of people with stone disease are different from healthy individuals in ways that may exacerbate stone formation. The majority of kidney stones are composed of calcium oxalate, and the gut bacteria in stone formers may be causing the increased levels of oxalate that we measured in the patient’s urine. Stone formers that are exposed to antibiotics more often also carried antibiotic-resistant bacteria within them, indicating previous antibiotic exposure damages the beneficial microbial ecosystem. When we investigated medical devices (ureteral stents) that are used in stone patients which can become infected with bacteria, antibiotic use did not prevent the presence of harmful bacteria on the devices, or in the bladder. We also found that the stent microbiota was different if the patient had diabetes, IBS/IBD, and other comorbidities. These factors should be considered in future stone patients that require ureteral stents and we believe the standard of care of antibiotics needs to be modified. Using a model of stone disease in fruit flies, we determined that beneficial bacteria could protect against stone formation, and that harmful bacteria in the urinary tract may be making stone disease worse. Future research could lead to the development of effective probiotics against kidney stones. Overall, we have found that bacteria are intimately involved in stone formation — some harmful bacteria present in stone formers encourage stone formation, while beneficial bacteria in healthy people can be protective. This condition is increasing in prevalence, and we need better solutions to prevent it — this work illustrates that bacteria may be the key to future kidney stone treatment.
Co-Authorship Statement

The experiments and data analyses within this thesis were predominantly carried out by Kaitlin Al with supervision from Jeremy Burton. The manuscripts presented within were primarily written by Kaitlin Al. Exceptions are listed below:

Chapter 2: Evaluation of sampling and storage procedures on preserving the community structure of stool microbiota: A simple at-home toilet-paper collection method

Kaitlin Al, Jordan Bisanz, and Jeremy Burton conceived the experiment, with input from Gregor Reid and Gregory Gloor. Kaitlin Al collected and processed all samples, which were sequenced at the London Regional Genomics Centre (LRGC) by David Carter. Kaitlin Al performed data analyses with input and code contributed from Greg Gloor, Jordan Bisanz, and Jean Macklaim.

Chapter 3: The microbiome at multiple body sites in a kidney stone patient population: revisiting the role of Oxalobacter formigenes in stone formation

Kaitlin Al, Jeremy Burton, and Hassan Razvi conceived and designed the study, with input from Gregor Reid. Participant enrollment, follow up, and sample collection was completed by Kaitlin Al with input from Linda Nott, Hassan Razvi, and John Denstedt. Patient clinical files were reviewed by Kaitlin Al, Linda Nott, and Patricia Rosas-Arellano. Sample processing was completed by Kaitlin Al, and 16S rRNA gene sequencing samples were sequenced at LRGC by David Carter. Whole shotgun metagenomic sequencing was performed at The Centre for Applied Genomics by Sergio Pereira. Kaitlin Al performed sequencing analysis with input and code contributed from Gregory Gloor, Daniel Giguere, and Benjamin Joris. HPLC of urine samples was performed by Kaitlin Al and John Chmiel, using protocols designed and validated by John Chmiel. Kaitlin Al performed analysis of HPLC data.

Chapter 4: Characterizing the microbial communities associated with ureteral stents
Kaitlin Al, Jeremy Burton, and Hassan Razvi conceived and designed the study with input from Gregor Reid. Participant enrollment and sample collection was completed by Kaitlin Al and Patricia Rosas-Arellano, with assistance from the St. Joseph’s Urology Clinic nurses, Hassan Razvi, John Denstedt, Blayne Welk, Stephen Pautler, and Linda Nott. Kaitlin Al and Patricia Rosas-Arellano reviewed patient clinical files. Dr. Todd Simpson from the Western University Nanofabrication facility performed SEM and X-ray diffraction spectroscopy analysis. David Carter at LRGC performed 16S rRNA gene sequencing. Kaitlin Al analysed sequencing results with input and code contributed from Gregory Gloor. Gregory Gloor and Gregor Reid provided assistance with interpreting results.

Chapter 5: Utilization of a *Drosophila melanogaster* model of stone formation to explore host-microbe interactions in nephrolithiasis

Kaitlin Al and Jeremy Burton conceived the experiments with input from Gregor Reid, Hassan Razvi, Jennifer Bjaevoic, Ryan Chanyi, and David Holdsworth. Initial BS168 + oxalate growth curves were performed by Ryan Chanyi, which were then repeated and analyzed by Kaitlin Al. Kaitlin Al performed all *Drosophila melanogaster* (DM) experiments. Jennifer Bjaevoic imaged the DM + UTI89 fly cohort with polarized light microscopy, which was analyzed by Kaitlin Al. Kaitlin Al performed cell culture experiments and analyses with input from Jennifer Bjaevoic. Danny Poinapen and Jaques Milner designed and fabricated the µCT scanning apparatus. Danny Poinapen completed µCT scans with input from Kaitlin Al, Joanna Konopka, Chris Norley, Jaques Milner, and David Holdsworth. Brendan Daisley provided technical assistance with the qPCR experiments and their subsequent analysis. David Carter from LRGC performed the sequencing. Sequencing results were analysed by Kaitlin Al using custom code and code contributed from Gregory Gloor.
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Chapter 1

1 General Discussion

Kidney stone disease, or nephrolithiasis, is one of the most prevalent urologic pathologies. The disease has existed in parallel with civilization: it is referred to in the Hippocratic Oath, and stones have even been discovered in ancient Egyptian mummies. Various different compositions of kidney stones exist, each with unique etiology and associated risk factors. Despite a wealth of research existing on the mechanisms of stone formation, much still remains elusively idiopathic.

In the field of urology, bacteria have traditionally been thought of only in the context of infection, but we now know there to be a microbial community present in the healthy urinary tract. The microbial ecosystems living on and in us, as well as their genes and products are collectively referred to as the microbiome. The extensive human microbiome research field has uncovered previously unknown relationships with various states of systemic health and disease; however, consideration of the microbiome in urological conditions is only just emerging. The gut microbiome may indirectly affect stone disease by dictating what solutes ultimately end up in the urine, whereas the urinary microbiome may have a direct effect at the site of stone formation. Herein we explore how the human microbiome as a whole may impact stone disease, from the relative risk factors, to the inception of stone formation, and the role of bacteria in future disease treatment.

1.1 Kidney stone disease

Nephrolithiasis affects approximately 10% of the North American population; prevalence rates appear to be rising and have more than doubled over the last four decades in adult Americans (Pearle et al., 2005). Nephrolithiasis also has an extremely high rate of recurrence, estimated at 50% within 5 years (Pearle et al., 2005, Stamatelou et al., 2003). These features compound the burden of the disorder when the patient morbidity and economic onus are also considered. While some stones may be asymptomatic and detected incidentally, others can cause extreme renal colic and complications leading to emergency room visits, hospitalization, and surgical intervention (Khan et al., 2016). The loss of work
and treatment costs due to urolithiasis were associated with an estimated annual expenditure of greater than $5 billion in the United States (Pearle et al., 2005).

Kidney stones are composed of organic and inorganic crystals combined with various urinary macromolecules (Rodgers, 2017) and can form in the renal pelvis and calyces either freely or bound to renal papillae (Figure 1). These calculi result from supersaturation of urinary solutes that can precipitate and crystallize, leading to crystal aggregation and stone growth. Many stones begin as Randall’s plaques, which are calcium phosphate deposits found on the tips of renal papillae and are often associated with recurrent stone formation (Daudon et al., 2015; Kim et al., 2005; Kuo et al., 2003; Matlaga et al., 2007). Of the various compositions of kidney stones, calcium-based stones are by far the most common, comprising more than 80% of all stones, the majority of which are calcium oxalate (CaOx) (Moe, 2006). Less common stone compositions include uric acid (5-10%), struvite (5-15%), cystine (1-2.5%), and drug-induced stones (<1%) (Moe, 2006).

The propensity for stone formation differs by sex, age, geography, race, and body mass index (BMI) (Scales Jr. et al., 2012). Historically stone disease was between 2-3 times more common in men than women (Soucie et al., 1994), however in recent years this disparity has narrowed significantly (Ordon et al., 2015; Scales Jr. et al., 2007; Strope et al., 2010). In Ontario, it was found that the rate of kidney stone procedures performed per year between 1991 and 2010 increased by approximately 48%, which was accounted for mainly by an increase in procedures performed on women (Ordon et al., 2015). This rise in disease burden in women may be due to inflating rates of obesity, decreased fluid intake, and other lifestyle changes that are associated with risk of stone formation, as discussed later (Strope et al., 2010).

Kidney stone data are collected in different fashions, but among countries with age-stratified stone prevalence data there is a rise and fall pattern where incidence normally peaks around age 50, and subsequently decreases (Romero et al., 2010). Geographical variation in stone disease in the United States reflects environmental risk factors; stone prevalence is increased in hotter climates leading to decreased hydration status and more concentrated urine, as well as areas with high sunlight and increased Vitamin D production. This manifests in the southeastern states, where stone prevalence is highest, compared to the lowest rates in the
northwest (Romero et al., 2010; Soucie et al., 1994). In the US, race impacts stone prevalence whereby rates are highest for non-Hispanic white individuals, followed by Hispanics, African Americans, and Asian-Americans (Scales Jr. et al., 2012). The reason for race differences has not been determined but may pertain to diet and lifestyle.

Interestingly, conditions known to be associated with kidney stones include obesity, diabetes, metabolic syndrome, and cardiovascular disease. In a prospective study of over 240 000 individuals, Taylor et al. (2005) determined weight, weight gain since early adulthood, waist circumference, and body mass index to all be positively associated with the risk of incident kidney stones. The mechanisms of these associations are unclear but may be related to increased urinary excretion of calcium, oxalate, and uric acid in larger body sizes, which may lead to stone formation (Lemann Jr. et al., 1996). Hyperinsulinemia and diabetes are also known to be associated with stone disease: Lieske et al. (2006) determined that the probability of being diabetic was increased by 22% when comparing kidney stone formers to a group of non-stone formers after adjusting for age and BMI. This increased risk may be due to altered urine composition and lower urine pH which can result in uric acid crystalluria, a risk factor for both calcium and uric acid stone formation (Daudon and Jungers, 2007; Khan et al., 2016; Lieske et al., 2006). Metabolic syndrome traits (abdominal adiposity, increased serum triglyceride level, decreased serum high-density lipoprotein cholesterol level, hypertension and impaired glucose tolerance) was determined to significantly increase the odds of self-reported kidney stone disease, and the presence of four or more traits was associated with an approximately two-fold increased odds (West et al., 2008). Kidney stone formers often have risk factors associated with atherosclerosis, and several studies have shown cardiovascular disease to be associated with stone formation (Alexander et al., 2014; Ferraro et al., 2013; Rule et al., 2010). Alexander et al. (2014) found that after adjusting for confounders, individuals that had at least one kidney stone were at higher risk for both myocardial infarction and stroke, but the reason for this correlation is not known.

1.2 Mechanisms of stone formation

Stone formation initiates by urinary salt supersaturation: when a solute (salt) is added to a solution (urine), it will dissolve until a certain concentration is reached, beyond which the solution is saturated and the solute crystallizes (Aggarwal et al., 2013). The concentration of
saturation can be altered based on the presence of crystallization inhibitors and promoters that are normally found in urine, however urine is generally reported as metastable with respect to most stone components (Ratkalkar and Kleinman, 2011). Despite this, if the supersaturation raises high enough, crystal nucleation will still occur. Crystal nucleation occurs when free solute ions dispersed in the solvent (urine) change to a solid phase and associate into particles (Ratkalkar and Kleinman, 2011; Rodgers, 2017). This nucleation can occur in free solution, on cell surfaces, or in other micro-environments such as areas within the nephron (Figure 1) (Evan et al., 2003; Khan et al., 2016). Following nucleation in solution, crystals will aggregate into larger particles, the surface of which can further act as a nucleation site for new crystals. The aggregation process can be promoted by various compounds and cell-derived material such as proteins, lipids, polysaccharides, and glycoproteins which can eventually form the organic matrix between crystals in the stone (Hess et al., 1993; Khan and Hackett, 1993).
A) Stones can form in the renal calyces or pelvis and can be either free-floating or attached to renal papillae. Struvite stones can often develop into a staghorn morphology, where the stone body occupies the entirety of the renal calyces and pelvis. Most stones less than 5 mm in size can pass on their own, but those > 5 mm may become lodged in the ureter or bladder. The length of the ureter shown is not to relative scale, the average adult ureter is between 25-30 cm. B) The induction of a stone nidus is hypothesized to occur in two ways. In the fixed-particle mechanism of Randall’s plaques, calcium phosphate precipitates in the basement membrane of the loop of Henle, aggregating in the interstitium and eventually eroding into the renal pelvis. In the free-particle mechanism of Randall’s plugs, crystals form in the renal tubules and aggregate, plugging the terminal collecting ducts. When both plaques and plugs are exposed to the renal pelvis, they act as a nidus of further crystal deposition from supersaturated pelvic urine. Image templates from Servier Medical Art by Servier were used and modified under the Creative Commons Attribution 3.0 Unported License.
The two dominant models of stone formation were first described by Randall as plu-
gles and plaques (Figure 1B) referring to the free and fixed particle mechanisms respectively (Randall, 1937; Randall, 1940). In the free particle model, urinary supersaturation leads to crystal formation in the renal tubules, which aggregate and plug the terminal collecting duct’s opening to the renal pelvis (Khan and Canales, 2015). These plugs are then exposed to the pelvic urine and act as a nidus for further crystal deposition and stone formation. In the fixed particle theory of Randall’s plaques, calcium phosphate (CaP) crystals precipitate in the basement membrane or vasa recta of the loop of Henle (Evan et al., 2003; Matlaga et al., 2006; Stoller et al., 2004). These crystals then aggregate in the interstitium, accumulating and eventually eroding through the papillary surface. When exposed to the pelvic urine, the CaP crystals act as a nidus for CaOx crystal deposition. Indeed, several autopsy studies have shown CaOx stones to be formed attached to Randall’s plaques, while other studies have validated that CaP is often found at the nidus of CaOx stones (Haggitt and Pitcock, 1971; Meyer et al., 1975; Randall, 1940; Stoller et al., 1996; Weller et al., 1972; Xie et al., 2014). An examination of both modes of stone formation demonstrates various sites where bacteria could form a nidus in the kidney.

1.3 Kidney stone composition

As stated above, the most common kidney stones are calcium-based in the form of CaOx (75%) and CaP (5-10%) (Moe, 2006). The former can exist as CaOx monohydrate (COM) or dihydrate (COD). The COM crystals have a characteristic “dumbbell” appearance and form the most thermodynamically stable kind of stone (Alelign and Petros, 2018; Coe et al., 1992; Khan and Hackett, 1986). COD is seen less often than COM in clinically relevant stones, and forms crystals with tetragonal “envelope” appearance (Daudon et al., 2004; Khan and Hackett, 1986). Pure CaP stones are rare, but the crystals are the most abundant in mixed stones (Coe et al., 1992). CaP is most commonly found in the form of apatite (Ca_{10}[PO_4]_6[OH]_2) and brushite (CaHPO_4·2H_2O) (Coe et al., 1992; Siener et al., 2013b). Risk factors for all calcium stones include hypercalciuria and hypocitraturia; hyperoxaluria and hyperuricosuria are risks for CaOx stones while urinary pH > 7.5 is a risk for CaP stones (Siener et al., 2013b).
Uric acid (UA) stones typically account for between 5-10% of all stones, with increased prevalence among patients with obesity, diabetes, and metabolic syndrome (Cameron and Sakhaee, 2007; Pearle et al., 2005). The urate anion is soluble at pH > 5.3, so consistently acidic urine is a risk factor for UA stones (Cameron et al., 2012). Hyperuricosuria can be caused by elevated endogenous UA production (for example in conditions such as gout), increased purine catabolism (as occurs in patients receiving chemotherapy), consumption of a purine-rich diet, or chronic diarrhea leading to loss of bicarbonate and acidified urine (Abou-Elela, 2017; Landgren et al., 2017; Liebman et al., 2007; Tsimberidou and Keating, 2005).

Struvite or infectious stones represent between 5-15% of all stones (Pearle et al., 2005). The magnesium ammonium phosphate crystals that struvite stones are composed of typically have a “coffin-lid” appearance (Khan et al., 1986). These form in patients with urinary tract infections due to organisms that produce urease, most commonly Proteus mirabilis and less frequently Klebsiella spp., Pseudomonas aeruginosa, Staphylococcus saprophyticus, and Ureaplasma urealyticum (Thompson and Stamey, 1973). Urease cleaves urea to ammonia and CO₂, which elevates urinary pH and causes precipitation of the magnesium ammonium phosphate hexahydrate crystals (Das et al., 2017). These stones are approximately three times more common in women than men, presumably due to the higher prevalence of urinary tract infections in women (Han et al., 2015). Struvite stones commonly present as staghorn calculi, named for the horn-like shapes formed when the stones occupy the renal pelvis and extend into the calyces (Figure 1A) (Preminger, 2005).

Cystine stones comprise less than 2% of all stones and unlike other compositions are the result of an inherited defect in renal cystine transport affecting about one out of every 20 000 people (Knoll et al., 2005). Due to an autosomal recessive mutation on chromosome 2 in the dibasic amino acid transporter gene heavy chain subunit rBAT (SLC3A1) or its light chain subunit (SLC7A9) on chromosome 19, cystine is unable to be reabsorbed in the renal tubule and cystinuria occurs (Eggermann et al., 2012). At normal urinary pH cystine is insoluble and forms hexagonal crystals, though solubility is improved with alkalization. Traditionally cystine stones are quite hard and appear amber coloured and slightly opaque due to their sulphur content (Khan and Hackett, 1986). They tend to present as multiple, bilateral, large stones, (Han et al., 2015). Due to their genetic origin, cystine stones usually present earlier in life than other stone compositions (Harnevik et al. (2003) found that around 20% of patients
had their first stone before the age of 3) and have very high rates of recurrence (Shim and Park, 2014). No previous studies have investigated whether these stones or their high recurrence involves a bacterial component.

A small proportion of stones (>1%) are miscellaneous in composition and are drug induced (Han et al., 2015). Various xenobiotics including medications and environmental toxicants can directly crystallize in urine forming stones, or cause urinary metabolic abnormalities forming stones from conventional components such as calcium oxalate or uric acid (Daudon and Jungers, 2004). Perhaps the most notorious example of a lithogenic drug is indinavir-sulfate, a protease inhibitor used in HIV treatment, where incidence of stones in indinavir-treated patients has been reported to exceed 40% (Saltel et al., 2000). Other drugs known to crystallize in urine and have been found in stones include antibiotics such as ciprofloxacin and trimethoprim-sulfamethoxazole that are ironically commonly prescribed in urological practice (Albala et al., 1994; Cek et al., 2012; Chopra et al., 2000; Daudon and Jungers, 2004). Others include the diuretic triamterene (Daudon and Jungers, 2004; Ettinger et al., 1979; Gault et al., 1981), and cough and stimulant preparations composed of ephedrine and guaifenesin (Assimos et al., 1999; Bennett et al., 2004; Daudon and Jungers, 2004; Whelan and Schwartz, 2004). Melamine consumption can cause stones in acidic urine (Dalal and Goldfarb, 2011; Grases et al., 2009), and cadmium exposure is associated with significant risk of calcium and uric acid stone formation (Guo et al., 2018; Ramaswamy et al., 2016; Thomas et al., 2013).

In comparison to stones made of the crystallized drug itself, several xenobiotics can cause metabolically induced stones. For example, calcium and vitamin D supplements are known to induce hypercalciuria and calcium stones (Sorensen, 2014), vitamin C is an oxalate precursor and overdosing can lead to hyperoxaluria and calcium oxalate stone formation (Ferraro et al., 2016), and laxative abuse can lead to formation of ammonium urate stones (Dick et al., 1990). More generally, medications known to alter the urinary pH can also cause susceptibility to stones; acidic urine is a risk factor for uric acid stone formation, and alkaline urine can predispose to stones from urate salts and calcium phosphate (Abou-Elela, 2017; Daudon and Jungers, 2004; Moe, 2006). Although drug-induced stones are rare, they deserve consideration due to their preventable nature.
1.4 Kidney stone management and treatment

While stones can be removed surgically or in some cases dissolved as described below, their prevention is deemed most important because of the extremely high recurrence rate and the potential for stones to cause serious complications such as chronic kidney disease and end-stage renal disease (El-Zoghby et al., 2012; Rule et al., 2009).

In addition to the specific metabolic changes required to prevent stones detailed in Table 1, the simplest risk factor to control for in the management of kidney stone disease is low urine volume. A preventative strategy should involve increasing water intake to achieve a urine volume of at least 2.5 litres per day (Borghi et al., 1996; Pearle et al., 2014; Xu et al., 2015). This increased hydration can be accompanied by several dietary modulations to further lower the risk of stone formation. A balanced diet with reduced animal protein intake has been shown to protect against recurrence of calcium and uric acid stones (Han et al., 2015; Nguyen et al., 2001; Borghi et al., 2002). Reduced sodium intake can also decrease the risk of calcium stones since sodium prevents renal calcium reabsorption and therefore increases urinary calcium levels (Nouvenne et al., 2010). In general, increased vegetable and fruit consumption is beneficial for stone formers as components such as potassium can be metabolized into alkali and thereby raise urinary pH (Berg et al., 1992; Moe, 2006; Rose and Westbury, 1975). It is important for stone formers to not restrict calcium intake, as this decreases the amount of calcium available in the gastrointestinal tract to bind to dietary oxalate, which subsequently increases oxalate absorption and oxaluria (Jaeger et al., 1985; von Unruh et al., 2004). Similarly, since oxalate is present in many common (and otherwise healthy) foods, stone formers should only avoid those that are very high in oxalate and shown to increase urinary oxalate levels such as rhubarb, spinach, green tea, chocolate, nuts, strawberries, and wheat bran (Grases et al., 2006; Massey et al., 1993).

Other aspects of medical management of stone disease involve the treatment of renal colic, medical expulsive therapy, and stone dissolution therapy. Renal colic can occur when stones shift within the urinary tract, causing obstruction and/or hydronephrosis, and may be associated with hematuria, dysuria, urinary urgency and frequency, nausea, and vomiting (Teichman, 2004). Nonsteroidal anti-inflammatory (NSAID) drugs and opiates are the first line therapy for analgesia of renal colic, with an added benefit of NSAIDs being that they
reduce glomerular filtration rate (Clark et al., 2011; Portis and Sundaram, 2001). Medical expulsive therapy can involve the use of $\alpha$- blockers such as tamsulosin which can inhibit ureteral spasm and uncontrolled contraction, dilating the ureter and increasing the probability of spontaneous stone passage (Assimos et al., 2016). Stone dissolution therapy is generally only effective for uric acid stones, where alkalizing urine with potassium citrate (or other compounds) to pH $\geq 6.0$ with increased urine volume has been shown to partially dissolve the stones (Moran et al., 2002; Pearle et al., 2014; Teichman, 2004).
Table 1: Pathophysiology and associated treatment of nephrolithiasis

<table>
<thead>
<tr>
<th>Metabolic Anomaly</th>
<th>Associated Stone Composition</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypercalciuria</td>
<td>• Calcium oxalate • Calcium phosphate</td>
<td>• Decreased sodium and protein consumption</td>
</tr>
<tr>
<td>Hypocitraturia</td>
<td>• Calcium oxalate • Calcium phosphate • Uric acid</td>
<td>• Consumption of citrate (citrus fruits, potassium citrate)</td>
</tr>
<tr>
<td>Hyperoxaluria</td>
<td>• Calcium oxalate</td>
<td>• Reduced oxalate consumption • Avoidance of calcium restriction</td>
</tr>
<tr>
<td>Hyperuricosuria</td>
<td>• Uric acid</td>
<td>• Decreased consumption of purine-rich foods • Allopurinol</td>
</tr>
<tr>
<td>Cystinuria</td>
<td>• Cystine</td>
<td>• Increase urine pH • Increased urine volume • Thiol-based chelating agents (Andreassen et al., 2016)</td>
</tr>
<tr>
<td>Low urinary pH</td>
<td>• Uric acid • Cystine</td>
<td>• Potassium citrate • Sodium bicarbonate (Pinheiro et al., 2013)</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>• Struvite</td>
<td>• Antibiotics</td>
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</tbody>
</table>
Most stones less than 5 mm will pass on their own, but surgical management is required when stones are greater than 10 mm in diameter, cause severe colic, have not passed in four weeks, or cause other complications (Khan et al., 2016). The three most common methods of stone treatment are extracorporeal shockwave lithotripsy (ESWL), rigid or flexible ureteroscopy (URS), and percutaneous nephrolithotomy (PCNL). These procedures account for about 40-50%, 30-40%, and 5-10% of worldwide surgical stone treatments, respectively (Khan et al., 2016). The choice between treatment modalities depends on patient characteristics such as medical co-morbidities and anatomy, stone features such as location, radiodensity, size, and the experience of the treating physician.

ESWL is a non-invasive method of fragmenting calculi; acoustic shock waves are created from electrohydraulic, electromagnetic, or other generators and focused on the stone with a lens (Rassweiler et al., 2014; Elmansy and Lingeman, 2016). The stone is visualized with ultrasonography or fluoroscopy, and fragmented when the focused shockwaves pass through it and energy is released (Elmansy and Lingeman, 2016). The stone fragments then pass out of the body during urination, and success of the ESWL is usually assessed by ultrasonography or radiography in the following weeks (Chiang et al., 2017; Elmansy and Lingeman, 2016). ESWL is usually the first line treatment for stones < 1 cm in size, and treatment success rates are lower for larger stones, those located in the lower pole, and in shockwave resistant stones such as calcium oxalate monohydrate and cystine (Turk et al., 2016). ESWL success is also associated with lower BMI and skin-to-stone distance (McClain et al., 2013; Nakada et al., 2000; Pareek et al., 2005). General or regional anesthesia is utilized in ESWL procedures for analgesia and to minimize movement and the respiratory motion of the kidney (Eichel et al., 2004; Turk et al., 2016). ESWL has a low risk of complications and morbidities but can sometimes induce hematuria, infection, steinstrasse, and very rarely sepsis (Kelley, 1990). Interestingly, experiments performed almost thirty years ago demonstrated that bacteria suspended in urine lost viability when exposed to ESWL, and even more so when incorporated into agar beads mineralized with calcium carbonate crystals simulating stones (Reid, et al., 1990).

Stone-free rates are comparable for treatment with ESWL and URS; URS may achieve better stone-free rates with a single procedure whereas ESWL may require multiple, however ESWL has fewer complications (Turk et al., 2016). URS is minimally invasive and involves
retrograde passage of a rigid or flexible endoscope from the urethra proximally to the ureter and kidney, aided by fluoroscopy. Ureteral stones have higher surgical success rates from treatment with rigid ureteroscopes, whereas flexible ureteroscopes are better utilized for proximal stones and less anatomically accessible calyces (Turk et al., 2016; Wright et al., 2014). Lithotripsy during URS is most commonly achieved with the use of a holmium laser and has highest stone free rates in stones between 10-20 mm in size (Turk et al., 2016; Wright 2014). Spinal or general anesthesia is utilized in URS for analgesia and to minimize the visceral response to urinary tract dilation (Khan et al., 2016). Although URS complication rates are slightly higher than ESWL at 9-25%, most complications are minor and do not require intervention; the majority of complications following URS are secondary to the placement of a ureteral stent following the procedure which can cause morbidity and irritative voiding (Turk et al., 2016).

Percutaneous nephrolithotomy was first introduced in 1976 and is now the standard of care for large (>20 mm) or complex renal stones (Fernström and Johansson, 1976; Turk et al., 2016). The procedure is usually performed with the patient in prone position, where fluoroscopic imaging is utilized to aid endoscopic renal access via a posterior calyx through an initial needle puncture of the skin, muscle, and perineal fat (Khan et al., 2016; Vicentini et al., 2009; Turk et al., 2016). Once the access tract has been dilated, various instruments may be inserted and utilized for stone removal including lithoclast and ultrasound probes, graspers, and baskets. Overall, the stone free rate of PCNL is between 80-90% but it is more invasive and thus associated with more complications than both ESWL and URS. The most common complications postoperatively are fever, bleeding requiring transfusion, and stent-associated morbidity, but more rarely PCNL can lead to sepsis, pneumothorax, embolization, or injury to other organs (Turk et al., 2016).

In summary, management of renal calculi can be expensive, complicated, and in many patients, it does not prevent recurrence; these painful events adversely and multifactorially affect quality of life. Thus, investigation into the potential role that bacteria may play in stone formation, prevention, and treatment is required in order to decrease the burden of this disease.
1.5 Evidence for microbial involvement in nephrolithiasis

The microbiota is the term used to describe the microorganisms present in a given environment (Whiteside et al., 2015). The term microbiome encompasses the biotic and abiotic factors in an environment, including the genes and genomes of the microbiota, as well as the products of the microbiota and the host (Whiteside et al., 2015). In the human body, the largest collection of bacteria resides in the colon (Sender et al., 2016). Although previously considered sterile, it is now known that the healthy urinary tract also harbours a unique microbiome that is distinct from the communities of the gut and vagina (Hilt et al., 2014; Lewis et al., 2013; Wolfe et al., 2012). With the advent of next generation sequencing technologies, extensive research has been done on the role of the microbiome in human health.

We now know that gut microbial dysbiosis can impact host metabolism (Larsen et al., 2010; Ley et al., 2006; Turnbaugh et al., 2006), immunity (Scher et al., 2013), the brain (Rhee et al., 2009), and even the heart (Gan et al., 2014). On the other hand, consuming beneficial microbes such as those present in probiotics and fermented foods may lower the risk of urinary conditions such as bladder cancer for reasons not yet uncovered (Larsson et al., 2008). In addition, there are some studies suggesting that fermentation can reduce dietary derived phytates and oxalates, potentially lowering the risk of stones (Al-Wahsh et al., 2005). Overall, the intestinal microbiome and barrier is important for not only preventing pathogens from entering the bloodstream, but also for adsorption of nutrients, drugs, and coordinating immune responses. A range of proteins are key to its protective barrier, and if those are damaged a range of metabolic and inflammatory diseases can occur (Lee et al., 2018).

To date, relatively little is known about the role of the gut and urinary microbiome in kidney stone disease. Several studies have identified differences in the gut microbiota of a small population of kidney stone formers compared to non-formers, and it has long been thought that gut colonization with Oxalobacter formigenes reduces oxalate kidney stone risk (Kaufman et al., 2008; Sidhu et al., 1998; Stern et al., 2016; Zampini et al., 2019). However, a closer investigation of the total human microbiome in nephrolithiasis as a whole is needed.
1.5.1 Oxalate and *Oxalobacter*

As discussed above, urinary oxalate levels are a major risk factor for calcium oxalate kidney stone formation (Curhan *et al.*, 2001). Blood oxalate levels are dictated by a combination of exogenous dietary consumption (Hatch and Freel 2005; Holmes *et al.*, 2001), as well as endogenous production by the liver (Baker *et al.*, 2004; Holmes and Assimos, 1998), erythrocytes (Jennings and Adame, 1996; Marengo and Romani, 2008), and the metabolism of ascorbate (Figure 2) (Knight *et al.*, 2006; Linster and van Schaftingen, 2007; Marengo and Romani, 2008). One approach to lowering urinary oxalate levels is to decrease dietary oxalate intake by eliminating high oxalate foods, however the effectiveness of this method is not well established (Liebman and Al-Wahsh, 2011). Oxalate absorption occurs in the gastrointestinal tract (GIT) through both paracellular and transcellular transport (Hatch and Freel, 2005; Hatch and Freel, 2008), and can also be excreted from the circulation into the GIT (Figure 2) (Freel *et al.*, 2006; Hatch *et al.*, 2011).

It has long been hypothesized that intestinal colonization by bacteria with oxalate-degrading capacity is inversely correlated with kidney stone risk. One such bacterium is the Gram-negative obligate anaerobe *O. formigenes*, which utilizes oxalate in the intestine as its primary carbon source (Allison *et al.*, 1985; Stewart *et al.*, 2004). There have been numerous studies and reviews relating to *O. formigenes*, and it is now considered a keystone microorganism of the healthy microbiome (Barnett *et al.*, 2016; Duncan *et al.*, 2002; Goldfarb, 2004; Hatch *et al.*, 2008; Knight *et al.*, 2013; Liebman and Al-Wahsh, 2011; Prokopovich *et al.*, 2007; Siener *et al.*, 2013a; Stewart *et al.*, 2004). However, until now, there has not been extensive research done on other bacterial types that may also be involved in human oxalate homeostasis. Some may degrade oxalate similarly to *O. formigenes*, while others may occupy important roles maintaining the intestinal barrier or altering oxalate transport in the GIT (Figure 2). We therefore need to consider the entire ecosystem including human intestinal barrier health for the handling of oxalate, and consequently, nephrolithiasis.

1.5.2 The microbiota and kidney stone disease

Several contemporary studies have looked to characterize the microbiota of kidney stones and of the stool of kidney stone formers (Barr-Beare *et al.*, 2015; Stern *et al.*, 2016; Tavichakorntrakool *et al.*, 2012; Wang *et al.*, 2014; Zampini *et al.*, 2019). Although struvite,
not calcium oxalate stones, are typically associated with infection, Barr-Beare et al. (2015) revealed that oxalate stones retrieved from pediatric patients contained small numbers of bacteria. It may be that if bacteria ascend to the kidney from the bladder, an inflammatory event occurs which when combined with elevated urinary oxalate could form a nidus for crystal deposition (Figure 2) (Tavichakorntrakool et al., 2012). Thus, unlike urinary tract infections which tend to harbour $10^5$ bacteria per mL urine, much smaller numbers may be sufficient to induce stone formation. In the first small study of the gut microbiota of kidney stone patients (23 patients, 6 healthy controls), Stern et al. (2016) detected higher levels of Bacteroides spp. and lower Prevotella spp. and beta diversity relative to healthy controls. Later in 13 stone patients and 13 healthy controls, Tang et al. (2018) showed similar findings with a decreased relative proportion of Eubacterium and bacterial beta diversity in the stone disease cohort. While interesting, these studies require a cautionary note: the tremendous heterogeneity of the gut microbiota can make drawing conclusions from so few participants extremely dubious, so it is evident that more studies are necessary. For now, the evidence suggests that members of the intestinal microbiota, not just O. formigenes, may affect stone disease.

1.5.3 Sulfate-reducing bacteria

An important factor connecting oxalate homeostasis, the microbiota, and intestinal barrier function is the sulfate anion transporter (Sat-1) protein, which is primarily expressed in the large intestine, but also in the liver and kidneys (Figure 2) (Schnedler et al., 2011). Albeit in a small cohort, a positive correlation has been shown for human variants of the SLC26A1 gene (encoding Sat-1), and risk of recurrent calcium oxalate stones, suggesting a biologically and clinically relevant role for this protein in nephrolithiasis (Dawson et al., 2013). This transmembrane antiporter can be positioned on the apical and basolateral membrane surface of epithelial cells, acting to transport oxalate into the intestinal lumen for excretion via the bidirectional exchange of sulfate anions (Figure 2) (Hatch and Freel, 2005). One would expect populations of sulfate-reducing bacteria in the gut microbiota to play a key role in the activity of this transporter through modulating the bioavailability of its influx substrate, sulfate. This effect may be most notably observed in autistic cohorts that demonstrate three-fold greater plasma oxalate levels, exhibit severe sulfate deficiencies, and harbour significantly higher levels of sulfate-reducing bacteria (e.g. Desulfovibrio spp.) in
comparison to controls (Konstantynowicz et al., 2012; Finegold, 2011). These traits when taken together are suspected to be involved in a greater risk of nephrolithiasis in these patients.
Dietary intake

- Fermented foods
- Antibiotics, pharmaceuticals
- and environmental sterilizers
- Dietary oxalate
Oxalate is consumed in the diet and produced endogenously in the liver; it can be excreted through the intestine or kidneys. In the gut lumen, beneficial microbes can break down dietary oxalate, and *O. formigenes* is also able to stimulate the excretion of oxalate anions into the gut from the circulation via the SLC26A family of transporters. In the bladder, the beneficial endogenous microbiota can become displaced by uropathogens during urinary tract infections. If uropathogens ascend to the kidney, an inflammatory event can occur leading to the development of a crystal nidus. Consumption of fermented foods and beneficial bacteria may promote microbiota robustness, aiding in oxalate-handling and decreasing risk of stone formation. Substances that deplete the microbiota such as antibiotics, environmental pollutants, poor Western diet and lifestyle factors could alternatively increase the risk of stone formation. Dietary oxalate could be both beneficial (by stimulating oxalate-degrading bacteria within the gut), or detrimental (if the oxalate-handling capacity of the gut microbiota is insufficient and serum oxalate levels become elevated). Image templates from Servier Medical Art by Servier were used and modified under the Creative Commons Attribution 3.0 Unported License.
1.6 Microbiome disruption and links to stone formation

1.6.1 Antibiotics

If the microbiome is involved in kidney stone disease, one would expect there to be a relationship between the disease and substances which disrupt or alter microbial populations, such as antibiotics. Disruptions to the microbiome have been well studied in metabolic syndromes such as obesity and diabetes, and there appears to be some correlation in the United States between where antibiotics are more frequently prescribed and these diseases, implicating the microbiome (Larsen et al., 2010; Turnbaugh et al., 2006; Turnbaugh et al., 2009). There also appears to be some overlap of high antibiotic prescription and nephrolithiasis in the south-eastern United States (Mandel and Mandel, 1989; Fisang et al., 2015; Hicks et al., 2013). It has been suggested that these “belts” of stone disease are related to dehydration; urinary solute precipitation is accelerated with decreased urine volume. Similarly, it has been suggested that the rise in kidney stone disease may be associated with global warming (Mandel and Mandel, 1989; Romero et al., 2010). Due to these multifactorial risks, it is difficult to ascertain whether these are just generally ailing populations, or whether their microbiomes have in fact been disrupted by antibiotic use, leading to stone formation and metabolic dysfunction.

In a more direct fashion, a recent study by Tasian et al. (2018) showed significantly increased odds of urinary stone disease with the use of five classes of antibiotics. In an impressive study looking at over 285,000 healthy controls and nephrolithiasis patients, they determined the association between 12 oral antibiotic classes and stone disease. Specifically, significantly increased odds of stone disease were associated with sulfas, cephalosporins, fluoroquinolones, nitrofurantoin/methenamine, and broad-spectrum penicillins. These effects were most significant with recent exposure and use at younger ages.

The incidence of nephrolithiasis in children also appears to be increasing rapidly, specifically in calcium-based calculi (Cameron et al., 2005). The reasons why this is occurring in children are unclear; it has been suggested that this is related to increasing BMI, salt consumption, decreased calcium and water intake, though perhaps it is due to the use of antibiotics in this young population (Sas, 2011). A mouse study modelling paediatric antibiotic treatment with either beta-lactam or macrolide antibiotics demonstrated altered
host and microbiota development, with a decrease in the relative abundance of oxalate metabolism genes, and fecal oxalate levels (Dethlefsen and Relman, 2011). Recent studies also suggest nephrolithiasis is up to four times more common in children with asthma, a condition that in turn has links with early childhood antibiotic use and microbiome dysbiosis (Nobel et al., 2015). Asthma is often treated with steroids, and glucocorticoid-mediated alterations on gut microbiota are known to occur (Kartha et al., 2017).

In adults, patients treated with antibiotics for Helicobacter pylori had decreased detection of O. formigenes (Kelly et al., 2011; Kharlamb et al., 2011). Of four O. formigenes strains tested against commonly used antibiotics, all were resistant to amoxicillin, amoxicillin/clavulanic acid, ceftriaxone, and vancomycin and at least one strain to nitrofurantoin (Lange et al., 2012). This would imply that the organism would survive administration of these antibiotics, but it does not rule out the depletion of supportive or symbiotic bacterial types.

Many mechanisms could be leading to these observed relationships with antibiotics and stone disease. If antibiotics increase stone formation, it could be that they are depleting other members of the gut microbiome that degrade and maintain oxalate homeostasis. Alternatively, direct crystallisation of various antibiotics can occur, and this precipitation in the kidney may act as a stone nidus (Chopra et al., 2000). Finally, the over- and misuse of antibiotics could be leading to antibiotic resistant uropathogens in the bladder which may ascend to the kidney, inciting inflammation and stone disease. The microbiome studies of the future will have to better control for antimicrobial substances (most recent usage, antibiotic class, use of sanitizers and detergents), as some of these compounds can have extremely long-term effects on the microbiome (Huang et al., 2015; Korpela et al., 2016).

1.6.2 Environmental damage to the microbiota

In addition to antibiotics, Western society is, in general, heavily medicated and bombarded with pharmaceuticals on a daily basis; in the United States, an average of over 11 prescriptions were filled per person per year in 2011 (Nash, 2012). This reflects consumption of prescription or over-the-counter medication, but also inadvertently through drinking water (Khan and Nicell, 2015). It is now known that drugs originally developed to target human cells rather than microbes can alter the microbiome, and while many such drugs often induce
gastrointestinal side effects, the direct effect on the microbiome is rarely investigated. An exception to this, Maier et al. (2018) completed an extensive screening of more than 1000 non-antibiotic marketed drugs against 40 representative gut bacterial strains. They found that almost a quarter of the tested drugs inhibited growth of at least one of the tested strains (Maier et al., 2018).

Similar trends exist with other environmental pollutants such as pesticides and industrial chemicals. These compounds can persist in the environment thereby exposing humans through dietary consumption, allowing direct interaction with the microbiota in the gastrointestinal tract (Jin et al., 2017). Work by Defois et al. (2018) examined a panel of persistent organic pollutants in chemostat fermenters and determined that all chemicals tested shifted fecal microbial composition and metabolic activity, with the potential to induce a pro-inflammatory state in the gut.

Many lifestyle factors of Western society are also significantly damaging to the human microbiome. In the current age, stress is recognized to be a significant burden; in the United States, the Center for Disease Control and Prevention estimates stress to account for around 75% of all doctor’s visits (Simmons and Simmons, 1997). Chronic psychosocial stress is known to have negative effects on the immune system and microbiome, which can ultimately result in diseases such as inflammatory bowel disease and colitis (Duffy et al., 1991; Gao et al., 2018; Langgartner et al., 2018). Kidney stone disease is also known to be impacted by stress and may be accounted for by the chain reaction of negative and microbiome-altering lifestyle factors that can be associated with stress such as poor diet, dehydration, and weight gain (Miyaoka et al., 2012).

A sedentary lifestyle not only contributes to obesity but can also directly impact the microbiome. Exercise has been found to directly alter the microbiome composition and metabolic function independently of diet and can increase the bacterial production of beneficial short-chain-fatty acids important in maintaining intestinal barrier function (Allen et al., 2017; Allen et al., 2018). Importantly, light exercise can significantly decrease the risk of stone formation (Sorensen et al., 2014), and short-chain fatty acids as well as the bacteria responsible for their production have been shown to be depleted in the gut of kidney-stone patients compared to matched healthy controls (Liu et al., 2019; Ticinesi et al., 2018). A
potential mechanism for this relationship which warrants further investigation may be that the bacterially produced short-chain fatty acids, through maintaining intestinal barrier integrity, decrease the paracellular absorption of intestinal oxalate into circulation (Hatch and Free, 2005; Vaziri et al., 2016).

The classical Western diet high in refined foods, animal protein, fat, and sugar, is known to cause negative alterations to the gut microbiome, including changes to bacterial abundance and community structure as well as decreased overall diversity (Turnbaugh et al., 2006; Turnbaugh et al., 2008). As discussed above, direct relationships between diet and stone formation have been established (for example, animal protein consumption and uric acid stone formation), and studies on the Western diet have shown it to promote risk factors for nephrolithiasis such as calcium oxalate crystalluria (Siener and Hesse, 2002).

Alterations to the colonic microbiome can affect the intestinal environment at various levels, and lead to gut permeability (Marchesi et al., 2016). This can directly increase the absorption of oxalate and allow inflammatory bacterial components to enter the body (Hatch and Freel, 2008). Alternatively, the repetitive insults against the microbiome through the average 21st century Western lifestyle can lead to indirect susceptibility to stone formation via collateral damage, depleting us of the protective microbial diversity we once had (Smits et al., 2017).

1.7 Microbiome restoration therapies

While the evidence of bacterial involvement in kidney stones is still emerging, it looks as if future preventative therapies will need to account for bacteria playing a role in the pathology of the disease. If childhood antibiotic use is truly determined to be a contributor to the condition, an objective of future treatments might be to cultivate or reacquire a beneficial microbiome. We obtain most of our bacteria externally, starting from our mothers, followed by other family members and then through our food and environment. In fact, while many factors determine the composition of the gut microbiome, diet has been shown to change its composition in as little as two days (David et al., 2014). Thus, it may be possible to resurrect or restore the microbiota of children exposed to antibiotics using beneficial microbes, before it moves to an intransigent profile. While high dietary oxalate has been associated with the risk of kidney stones in adults, it may be that it also facilitates propagation of oxalate-degrading organisms. Studies suggest that the microbiota is an important factor allowing
humans to quickly adapt to altered diets, facilitating dietary diversity (David et al., 2014; Zimmermann et al., 2005). Thus, perhaps a compromise can be achieved whereby a diet can facilitate oxalate-degrading bacteria but not induce urolithiasis. In addition to dietary alterations, other approaches may be utilized to restore a beneficial and protective microbiome against stone disease.

1.7.1  **Oxalobacter replacement**

As detailed above, there are many studies which support the potential role that *O. formigenes* plays in degrading oxalate and enhancing its intestinal secretion; epidemiological evidence also supports its existence in ‘healthy’ people (Duncan et al., 2002). Studies have investigated the use of *Oxalobacter* as a probiotic, but surprisingly there have not been many studies in large cohorts that include *O. formigenes* supplementation while simultaneously controlling dietary oxalate intake (Duncan et al., 2002; Jairath et al., 2015). Although some stability and delivery studies have been performed on *O. formigenes*, its relatively limited use in human trials may be attributable to regulatory concerns when delivering a live microorganism without a history of safe use in foods for humans (Ellis et al., 2015). While the species is considered a strict anaerobe, it does appear to exhibit tolerability to bile salts and low pH, indicating *O. formigenes* could survive through the harsher regions of the GIT (Duncan et al., 2002). The *Oxalobacter*-containing probiotic Oxabact® is currently utilized for oxaluria and may be one such product urologists lean on in the future of kidney stone treatment and prevention.

1.7.2  **Probiotics**

Some species of lactic acid bacteria are also able to degrade oxalate, though to a much lesser degree than *O. formigenes* (Miller and Dearing, 2013). Studies have shown both positive and negative outcomes when testing non-*Oxalobacter* probiotic strains as therapy for patients with kidney stones (Abratt and Reid, 2010; Campieri et al., 2001; Lieske et al., 2010; Okombo and Liebman, 2010). It is important that probiotic studies be evaluated on a strain, dose, and format basis. It is not yet possible to determine whether this potential treatment has value due to the small numbers of patients, lack of follow-up, and broad preparations that have been used in these studies thus far (Abratt and Reid, 2010; Campieri et al., 2001; Lieske et al., 2010; Okombo and Liebman, 2010). Although yet inconclusive, the concept has merit
and deserves further investigation. Not only might some probiotic strains degrade dietary oxalate before absorption, but they could also stabilize intestinal barrier integrity - a factor known to be critical in oxalate absorption into circulation and the luminal secretion of oxalate during host-mediated detoxification (Del Piano et al., 2012).

### 1.7.3 Engineered microbial solutions

Oxazyme® is a non-systemic orally delivered drug composed of recombinant oxalate decarboxylase for the treatment of primary hyperoxaluria. *In vitro* studies have shown that Oxazyme® can degrade oxalate in both simulated gastric and small intestinal environments, acting as an intercept therapy for the management of dietary oxalate prior to absorption (Mufarrij et al., 2013). However, it remains to be seen how often the enzyme has to be taken, when and at what concentration, and what impact it has on the microbiome and long-term risk of urolithiasis. *Bacillus subtilis* contains the oxalate decarboxylase gene *Yvrk* and has also been investigated for its functionality in oxalate nephrolithiasis treatment (Tanner and Bornemann, 2000). An *Escherichia coli* strain expressing the *Yvrk* from *B. subtilis* was developed and successfully degraded oxalate *in vitro*; purified enzyme from the recombinant *E. coli* showed oxalate degradation ability in a rat model of hyperoxaluria (Jeong et al., 2009; Lee et al., 2014). Similarly, a recombinant *Lactobacillus plantarum* was created to express and secrete oxalate decarboxylase which showed some efficacy in a rat model (Sasikumar et al., 2014a; Sasikumar et al., 2014b). While these studies demonstrate it is possible to design and engineer strains to produce oxalate-degrading enzymes, it is undetermined if they will promote oxalate’s intestinal secretion. There are likely other factors that impact how *O. formigenes* has adapted to handle oxalate in the colonized human gut, and it is unclear if recombinant probiotics or purified enzymes would provide long-term therapeutic value to oxalate nephrolithiasis patients.

### 1.7.4 Fecal microbiome transplantation

Animal studies have demonstrated that improved oxalate degradation may be achieved after whole community microbial transplants. Miller *et al.* (2016) showed that a fecal microbiota transplant (FMT) from the wild mammalian herbivore *Neotoma albigula* into laboratory rats, resulted in significant increases in oxalate degradation, an effect that persisted up to 9 months after the initial transplant. The selection of *Neotoma albigula* was important as it is uniquely
attuned to consume a diet high in oxalate (up to 9% dietary oxalate by weight), a phenotype which, as in all mammals, is conferred exclusively by the gut microbiota as opposed to mammalian enzymes. It will be interesting to see if this approach is developed and shown to be efficacious in humans for the treatment for oxalate kidney stone disease.

Currently, the primary use of FMT in humans is for recurrent *Clostridium difficile* infection, with reported success rates of up to 90% (Cammarota *et al.*, 2015; Jiang *et al.*, 2017; Kelly *et al.*, 2016; van Nood *et al.*, 2013). In Canada, FMT is performed on a routine basis for *C. difficile* infections and is proving to be one of the best treatment options (Chanyi *et al.*, 2017). The therapy is now being tested for treatment in extra-intestinal diseases, including metabolic syndrome, non-alcoholic fatty liver disease, and even multiple sclerosis (Borody *et al.*, 2013; Craven *et al.*, 2020; Henao-Mejia *et al.*, 2012; Vrieze *et al.*, 2012). Of interest, in a small metabolic syndrome study, patients who received an FMT from a lean donor often restored keystone microbes, including *O. formigenes* (Vrieze *et al.*, 2012). This treatment method may be promising for nephrolithiasis because of the known role of intestinal bacteria in oxalate degradation, barrier function, and oxalate secretion. While introduction of *O. formigenes* or other single-strain probiotics to a dysbiotic microbiome may only have short-term effects, an FMT could show promise as a more potent form of microbiome modification and treatment. Such an approach will require a new inclusion criterion for the donor, namely that they carry the *Oxalobacter* organism or other similarly beneficial traits.

A potentially more regulated approach to the FMT would be strategic microbiome reconditioning (Allen-Vercoe, 2013; Petrof *et al.*, 2013). A dysbiotic microbiome from a diseased individual could be collected, restored for specific functions *ex vivo*, then reintroduced to the patient (Chanyi *et al.*, 2017). This could be achieved by culturing the original sample in fermenters, or chemostat systems that have been pulsed with specific substances in order to increase the relative abundance of bacterial groups of interest. As discussed above, patients with oxalate stones are often advised to limit dietary oxalate. This is controversial because some people with diets high in oxalates, such as vegetarians, are often not at an increased risk of developing stones, perhaps because their microbiome is well-adapted to oxalate processing. Culturing of a stone patient’s fecal sample in the presence of
oxalate may offer a way to restore oxalate-degrading species to higher abundance, without the risks of the dietary oxalate consumption. The benefit of such a fermenter system would be that stone patients are receiving modified autologous transplants, thereby minimizing the risk of receiving any unwanted phenotypes that sometimes occur in allogeneic transplants (Alang and Kelly, 2015).

1.8 In vivo models of stone disease

It is important to utilize models to explore the many mechanistic questions that remain in nephrolithiasis and stone formation research. Both vertebrate and invertebrate models exist, each with their own strengths and limitations: models in both rats and mice have been developed, as well as in porcine, canine, and fruit flies. All models will be briefly reviewed with emphasis on similarities and differences compared to human stone pathophysiology and anatomy.

1.8.1 Rodent models of stone disease

In rats, hypercalciuria, several models of hyperoxaluria, as well as struvite stones have been developed. Bushinsky and Favus (1988) first published their model of hypercalciuria from an inbred strain of Sprague-Dawley rats. The model was developed after four successive generations of rats were selected with hypercalciuria caused by an increased number of vitamin D receptors in the gut, kidneys, and bones leading to increased calcium absorption (Bushinsky and Favus, 1988; Krieger et al., 1996; Li et al., 1993; Yao et al., 1998).

Hyperoxaluria has also been induced in rats via intraperitoneal injection of sodium oxalate, dietary consumption of glycolic acid, ethylene glycol in drinking water, intraperitoneal injection of hydroxy-L-proline, dietary consumption of potassium oxalate, dietary vitamin B₆ deficiency, ileum resection, and Roux-en-Y gastric bypass (Canales et al., 2013; Gershoff and Andrus, 1961; Khan et al., 1992; O’Connor et al., 2005; Ogawa et al., 1990; Robinson et al., 1990; Tawashi et al., 1980; Wiessner et al., 2011). A struvite stone model was also developed in outbred Sprague-Dawley rats, whereupon zinc discs were implanted in the bladder followed by instillation of Proteus mirabilis, leading to subsequent bladder calculi formation (Olson et al., 1989).
In mice, hyperoxaluria is associated with the \textit{ob/ob} model, and has been induced by similar methods to the rat models (ethylene glycol, hydroxy-L-proline and glyoxalate), but genetic knockout models are more commonly available and utilized in mice (Amin et al., 2018; Khan and Glenton, 2010). For example, knockouts for oxalate transporter SLC26A1 have been shown to induce CaOx stones, others for the sodium-hydrogen exchanger regulator factor-1 (NHERF-1) have been shown to induce renal calcium phosphate crystals, and knockouts for the cystine transporter light chain subunit SLC7A9 induce cystinuria and cystine stones (Feliubadalo et al., 2003; Jiang et al., 2006; Weinman et al., 2006). Both mice and rats have quite disparate kidneys compared to humans as they are obviously significantly smaller and have unipapillate- compared to human multi-papillate structure, although microscopically they have similar components and the species are genetically around 90\% similar to humans (Mullins and Mullins, 2004; Tzou et al., 2016).

1.8.2 Porcine models of stone disease

The porcine models of stone disease have utilized dietary supplementation of hydroxyproline and gelatin to induce hyperoxaluria (Mandel et al., 2004; Patel et al., 2012). Anatomically porcine and human kidneys are both multi-papillary, with comparable urine concentrations and glomerular filtration rates (Sachs, 1994). Although a benefit in terms of their relevance to humans, the major limitation of the porcine model is the animal’s size and subsequent high cost of husbandry. Another significant limitation for the purposes of urological study in pigs is that urine collection can also be quite challenging, as several studies have reported missed data due to urinary tract infections and catheter problems (Kaplon et al., 2010; Patel et al., 2012).

1.8.3 Canine models of stone disease

Much like humans and unlike other experimental models, canines commonly suffer from highly recurrent, spontaneous kidney stones (Furrow et al., 2017). The breeds Bichon Frise, Miniature Schnauzer, and Shih Tzu are considered clinically at substantially higher risks than others for struvite, calcium oxalate, and apatite stone formation (Low et al., 2010). Based on the spontaneous nature of their stone formation, canines present an animal model that is easy to work with in the laboratory and appropriate for cross-sectional studies in pet breeds. Having said that, the high cost, stringent ethical standards, and smaller sample size studies
are a hindrance. In addition, canine kidneys are significantly smaller than that of humans (except in dogs with a height >70 cm (137 g vs 39 g)), and the glomerular filtration rate for canines is approximately 2-fold that of humans (Maurya et al., 2018; von Hendy-Willson and Pressler, 2011). The collecting system is also structurally disparate between humans and dogs, whereby dogs are unipapillary but multipyramidal (Pereira-Sampaio et al., 2009).

1.8.4 Drosophila melanogaster models of stone disease

The conception of urinary stones in Drosophila melanogaster (DM) was first described by Chi et al. (2010) and solidified as a model of nephrolithiasis by Chen et al. (2011). Since its advent, it is now one of the most utilized models of the disease, due to its numerous advantages (Miller et al., 2013). DM have a short life cycle of fewer than 60 days in a laboratory setting and reproduce prolifically, while husbandry costs are very economical. As invertebrates, experimentation on DM also rarely requires approval by organizational ethical review boards. These features all facilitate easy experimentation and rapid data generation. There is a wealth of genetic tools available in DM, and mutant fly lines are readily available and inexpensive from commercial and academic stock centres. While the anatomy of humans and DM are inherently different, there are many functional, structural, and genetic similarities between the DM Malpighian tubule and the human renal tubule, grounding DM as a valid model for the study of nephrolithiasis (Miller et al., 2013).

The renal system in DM is composed of nephrocytes and Malpighian tubules (MTs)- two functionally distinct organs (Figure 3A). The DM hemolymph is filtered by the nephrocytes which are located in a cluster near the heart and esophagus, in a process which mirrors the filtration of blood by podocytes within the glomerulus in vertebrates (Weavers et al., 2009). Nephrocytes were at one point referred to as “storage kidneys”, where waste products filtered from haemolymph are actively endocytosed and are thought to be stored for the insect’s lifetime or coordinatively released in times of excessive diuresis (Weavers et al., 2009). The MTs resemble the remainder of the human nephron and collecting duct, actively transporting ions and solutes from the hemolymph and producing urine in the tubule lumen (Miller et al., 2013). DM have a posterior and anterior pair of MTs which join at two common ureters between the mid- and hindgut. Despite their relatively small genome size on just four chromosomes, nearly 80% of renal transporters in humans have genetic orthologs in DM (Reiter et al., 2001).
Several models of stone formation exist in DM, including both dietary and genetic varieties. Hyperoxaluria and calcium oxalate stone formation can occur in flies’ MTs upon supplementation of ethylene glycol, sodium oxalate, and hydroxy-L-proline, as well as through genetic knockdown of dPrestin, the DM homolog of the anion exchanger SLC26A6 (Chen et al., 2011; Hirata et al., 2012). A model of xanthine stone formation was generated through the silencing of xanthine dehydrogenase, while disruption of the Uro gene for urate oxidase is being investigated with regards to uric acid stone formation (Ali, 2017; Chi et al., 2015; Lang et al., 2018).

Some of the limitations of the DM stone models include the differences in anatomy between flies and humans. Where humans have the glomerulus and nephron, flies have the distinct organs nephrocytes and Malpighian tubules. The separate alimentary canals for liquid and solid waste in humans differ from flies which have a common cloaca where the ureters join the gut. Finally, as an invertebrate lacking bone, DM have a fundamentally different mechanism of calcium homeostasis from humans (Miller et al., 2013).

Compared to the human microbiota, that of DM is extremely simplistic and of very low diversity. Specifically, the genera Lactobacillus and Acetobacter comprise upwards of 95% of the microbiota in DM (Figure 3B) (Wong et al., 2011; Blum et al., 2013). Although in some regards this is a disadvantage for direct comparability of DM to humans, it is useful in experimentation in order to deconstruct potentially complex polymicrobial effects. Taken together, this simple in vivo model is useful for understanding the effects of specific members of the microbiome and how they may impact stone disease.
Figure 3. *Drosophila melanogaster* excretory anatomy and microbiota

A) The nephrocytes filter DM hemolymph, and urine is produced in the lumen of the Malpighian tubules. DM have one anterior pair and one posterior pair of Malpighian tubules; each pair connects at a common ureter at the junction of mid- and hindgut. B) The DM microbiota (i) is dominated by the genera *Lactobacillus* (phylum *Firmicutes*) and *Acetobacter* (phylum *Proteobacteria*), in comparison to the more complex human gut microbiota (ii). Average bacterial phyla present in i) DM based on Wong *et al.* (2011) and ii) humans based on Li *et al.* (2014).
1.9 Methodologies to assess microbial communities

For around 350 years, microscopes have been used to evaluate microbial communities. When van Leeuwenhoek first visualized bacteria in water samples in the late 1670’s, he was able to characterize them by relative size and morphology (Bardell, 1982). Van Leeuwenhoek was later the first to describe human-associated microbiota in his microscopic surveys of human saliva (Bardell, 1982). Fast forward to today, and immense technical advancement has drastically improved the visualization of microbes. Optical microscopy is still a very useful tool when visualizing the diversity and spatial organization of bacteria in different environments, especially when combined with bacterial staining or labelling strategies (Tropini et al., 2018).

Historically, when studying the human microbiota, culture techniques were utilized to grow bacterial isolates in predetermined medium. However, upwards of 80% of bacteria that reside within us are fastidious with very specific and complex growth requirements, making them “unculturable” under standard lab conditions. Thus, the diverse microbial composition of the human gut was drastically underestimated (Wilson et al., 1996). For this reason, the development of molecular, culture-independent techniques has been paramount in understanding the human microbiome. Early studies of the microbiota involved the generation of clone libraries of the small subunit ribosomal RNA genes (16S rRNA) followed by Sanger sequencing of short inserts (Wilson et al., 1996). Denaturing gradient gel electrophoresis (DGGE) was also used but necessitates significant user skill and lacks sensitivity (Burton and Reid, 2002). Fluorescence in situ hybridization (FISH) can now be used to enumerate bacteria with flow cytometry based on the binding and subsequent fluorescence of complementary 16S rRNA sequence probes, but this is probe-dependent and cannot identify unknown species (Fraher et al., 2012; Namsolleck et al., 2004). PCR-electrospray ionization mass spectrometry (PCR-ESI-MS) is yet another molecular technique capable of characterizing microbial communities, involving mass spectrometry of PCR amplicons such that the composition of nucleotides is deduced and compared against a database (Ecker et al., 2008; Nickel et al., 2015). This technique appears to perform comparatively to 16S rRNA gene sequencing with shorter workflow times but has not been widely implemented in the microbiome field, and instead has found favour for clinical diagnostics (Peeters et al., 2016; Zhang et al., 2019). Now, the most commonly used method
in the microbiota research field is 16S rRNA gene sequencing; it has become a powerful tool, requiring PCR with carefully designed barcoded primers and sequencing adapters that facilitate massive parallel sequencing output (Figure 4) (Gloor et al., 2010).

Classical techniques such as Sanger sequencing are capable of sequencing the entire 16S rRNA gene length, but lack multiplexing capability; when utilizing next generation sequencing (NGS, for example with the Illumina platform as was used in Chapters 2, 3, 4, and 5 of this thesis), sequencing read length is limited. The bacterial 16S rRNA gene has regions of high conservation flanking 9 regions of hypervariability (V1-V9, Figure 4). Due to NGS’s shorter read lengths, the variable region of interest to be sequenced should be carefully chosen to optimize the resolution of the microbiota profiling (Soergel et al., 2012). Based on the GC content of variable regions within different bacterial genera, PCR amplification may not work optimally biasing the data (Alcon-Giner et al., 2017). Alternatively, the variable regions of different bacterial groups may not in fact be that “variable”, limiting differentiation at lower taxonomic levels (Alcon-Giner et al., 2017). Thus, the V-region primer selection should be mindful of the suspected bacterial populations found within the sampled environment. Primers 515F-806R targeting the V4 region are utilized in the Earth Microbiome project and throughout this thesis, and are capable of differentiating common genera within the gut upon paired end sequencing (Thompson et al., 2017). Comparatively, primers targeting the V6 region can provide resolution between species of Lactobacillus, and thus would be utilized in sequencing of vaginal samples or yogurt, for example (Thompson et al., 2017).

Through the early years of microbiota analysis, the Roche 454 method of pyrosequencing was favoured due to the ability to generate reads upwards of 500 bases in length, spanning multiple variable regions on the 16S rRNA gene (Caporaso et al., 2010). When the Illumina platform was first utilized in the human microbiota field in 2010, the sequence length was 75 bases, but modern incarnations of the Miseq and HiSeq systems can generate lengths > 600 bp and have superseded 454 as the favoured contemporary platform (DiBella et al., 2013; Hummelen et al., 2010; Salipante et al., 2014). Nascent long-read sequencing technologies (capable of generating read lengths of 10s to 100s of kilobases) including those developed by PacBio and Oxford Nanopore may soon replace 16S variable region amplicon sequencing altogether (Callahan et al., 2019; Dohm et al., 2020).
Where 16S rRNA gene amplicon sequencing is the classic microbiota sequencing, shotgun metagenomic sequencing surveys the entire genome and genetic material of all organisms present in a sample, as opposed to only the 16S rRNA gene in bacteria. Shotgun metagenomic sequencing was utilized in Chapter 3 of this thesis. Metagenomic sequencing is less susceptible to biases inherent in amplicon sequencing, can provide higher taxonomic resolution, and can capture information from host, bacterial, viral, and fungal DNA, as well as functional pathways present in a sample (Hillmann et al., 2018; Jovel et al., 2016). However, it is significantly more expensive than 16S rRNA gene sequencing for both sequencing platform and computational costs and has fewer computational tools and databases available for analysis (Gevers et al., 2012).

The bioinformatic analysis of NGS data is complex, time-consuming, and there is no standard methodology across the field. Datasets generated by high-throughput sequencing are compositional in nature, as there is an arbitrary “total” imposed by the sequencing instrument; although the reads are discrete counts, they represent just a sampling of the original genetic material present in the sample (Gloor et al., 2017). When microbiome data are not treated in the appropriate compositional manner, incorrect assumptions and conclusions can be drawn (for example, the difference between claims of changes in absolute vs. relative abundance). Quantitative Insights Into Microbial Ecology (QIIME) is a commonly used analysis toolkit originally developed for pyrosequencing datasets, which facilitates data visualization, diversity analysis, and simple statistics (Caporaso et al., 2010). Several other analysis tools have been developed to overcome challenges in microbiome data analysis, including ALDEx2, and compositional data analysis packages within R software (van den Boogaart and Tolosana-Delgado, 2008; Fernandes et al., 2013; McMurdie and Holmes, 2013; R Core Team, 2013). These sequencing and bioinformatic tools have been utilized throughout the chapters of this thesis in various patient and sample populations, with the aim of determining how the microbiota influences stone disease.
Figure 4. Schematic of 16S rRNA gene and Illumina primer design

A) The 16S rRNA gene has highly conserved (light blue) regions surrounding nine variable (dark blue) regions. B) The primers utilized in this thesis amplified base pairs 515-806 encompassing the fourth variable region, and contained an Illumina adapter (sequences shown), followed by four random nucleotides, one of sixteen unique 12-mer barcodes (not shown), and the forward and reverse primers (sequences shown).
1.10 Project scope and purpose

At the commencement of my doctoral studies, little was known about the gut or urinary microbiome’s role in nephrolithiasis beyond *O. formigenes* colonization and the weakly described association between stones and a history of urinary tract infections. Thus, this project sought to increase the understanding of how the microbiome as a whole impacts stone disease, with specific emphasis on non-infectious stones. The following chapters will address four objectives of this project.

Objective 1. Before first evaluating the microbiome of kidney stone patients, the second chapter of this thesis aimed to establish a standard method of collecting and storing fecal samples for the purpose of next generation sequencing (NGS). While previous studies in our lab have evaluated best practices for processing urinary samples, the most patient-amenable and highest fidelity method of collecting fecal samples had not previously been assessed. In Chapter 2 it was determined whether toilet paper collected at home would be appropriate for NGS analyses after experiencing temporal and temperature variability, as would be experienced through the Canadian Postal Service. Additionally, it was investigated whether or not preservation with a nucleic acid stabilization agent was necessary. The methodology determined here was then utilized for the clinical study in Chapter 3 of this thesis.

Objective 2. Chapter 3 involved a clinical study into the differences in the human microbiome at different anatomical sites in kidney stone forming patients and healthy control participants. This study is the first of its kind utilizing shotgun metagenomic sequencing to evaluate the entire genetic capability of the gut community, as well as 16S rRNA gene sequencing to characterize the bacterial communities present in the bladder from multiple time points throughout stone treatment, and the microbiota within the stone itself. We detected significant and novel alterations in both the urinary and gut bacterial communities between the cohorts at both a taxonomic and functional level, which may be directly implicated in stone disease.

Objective 3. Chapter 4 describes a second clinical study that was undertaken to characterize the microbial communities associated with ureteral stents in a cohort of kidney stone forming patients, as stent placement is an almost ubiquitous component of surgical kidney stone management. This study is the largest and most comprehensive of its kind to date. The results
show that bacteria are commonly associated with stents and can lead to encrustation. It was established that the urinary microbiota is often not representative of the microbiota on the stent surface and is not an appropriate biomarker of stent encrustation. Several patient attributes were associated with altered stent microbiota, and antibiotic use was determined not to impact the microbiota, nor the degree of stent encrustation.

Objective 4. The fifth chapter investigates how specific beneficial and pathogenic microbes impact stone formation utilizing in vivo and in vitro models of stone disease. This chapter includes further method development and establishment of novel imaging techniques, evaluation of the implications of uropathogen exposure, as well as determination of the therapeutic potential of a novel probiotic for the treatment of nephrolithiasis. Results demonstrate that different uropathogens have diverging implications for stone disease, and the bacterium Bacillus subtilis strain 168 has promising probiotic capacity, improving stone disease burden in our Drosophila melanogaster model.

Overall, through both discovery and hypothesis-driven research, the unifying theme of this thesis is the desire to understand how the microbiome impacts stone disease, from the relative risk factors, to the inception of stone formation, and how it may further impact treatment and be exploited in preventative therapy. I hope that this body of work and the resulting publications from my PhD will have translational impact on urological practice and improve our understanding of microbes in human health.

1.11 References


Chapter 2

2 Evaluation of sampling and storage procedures on preserving the community structure of stool microbiota: A simple at-home toilet-paper collection method

This chapter is adapted with permission (Appendix A) from:

2.1 Abstract

The increasing interest on the impact of the gut microbiota on health and disease has resulted in numerous human microbiome-related studies emerging. However, multiple sampling methods are being used, making cross-comparison of results difficult. To avoid additional clinic visits and increase patient recruitment to these studies, there is the potential to utilize at-home stool sampling. The aim of this pilot study was to compare simple self-sampling collection and storage methods. To simulate storage conditions, stool samples from three volunteers were freshly collected, placed on toilet tissue, and stored at four temperatures (−80, 7, 22 and 32 °C), either dry or in the presence of a stabilization agent (RNAlater®) for 3 or 7 days. Using 16S rRNA gene sequencing with the Illumina MiSeq platform, the effect of storage variations for each sample was compared to a reference community from fresh, unstored counterparts. Microbial diversity and composition were not significantly altered by any storage method. Samples were always separable based on participant, regardless of storage method suggesting there was no need for sample preservation by a stabilization agent, which may complicate sample collection for the study participant and lead to increase costs. In summary, if immediate sample processing is not feasible, short term storage of unpreserved stool samples on toilet paper offers a reliable way to assess the microbiota composition by 16S rRNA gene sequencing.


2.2 Introduction

The human body, specifically the intestinal tract, is the site of a vast array of metabolically active microbes, collectively referred to as the microbiome. Over recent years the accuracy and scale of microbiome studies have increased dramatically due to the availability of next generation sequencing, and it is now recognized that the gut microbiota has a role in human health and disease (Manichanh, 2006.; Turnbaugh et al., 2006). The most common method of assaying the gut microbiota is through a fecal sample, but unfortunately there is no standard protocol for sample collection or processing. A fresh sample with immediate DNA extraction is the gold standard for microbiome researchers, however immediate freezing at -80 °C has also been shown to maintain the microbial composition (Carrol et al., 2012; Fouhy et al., 2015). Unfortunately, these are often not viable sampling procedures for clinical or field studies, where -80 °C may not be available or at the very least would require an additional clinic appointment for study participants. Thus, altered sampling and storage methodologies are often utilized for the purpose of maintaining the microbial community composition throughout storage (Choo et al., 2015; Song et al., 2016).

Such factors can all contribute to additional study costs and reduced participant recruitment. Additionally, various methodologies can make cross comparison between studies difficult. The American Gut sampling methodology is the simplest developed protocol to date (McDonald et al., 2015). Participants use a swab to take a small amount of fecal material from a stool or soiled toilet paper and mail it to the laboratory for processing. Of note, the length of time in transit and temperature exposure for each sample is variable depending on the time of year and participant location. Others have suggested that nucleic acids from fecal bacteria should be preserved in hyperosmotic solutions containing agents to prevent their degradation (Voigt et al., 2015). We sought to determine if a method adapted from the American Gut Project would provide reliable 16S rRNA gene profiles, even if stored under different conditions modelling seasonal temperature changes experienced during postal shipment (Gilbert et al., 2014; McDonald et al., 2015).

Although the immediate processing or deep freezing of samples is preferred, research groups have utilized cold chain storage and various nucleic acid preservation agents designed to maintain the bacterial DNA and microbial community structure of fecal samples (Blekhman...
et al., 2016; Flores et al., 2015; Fouhy et al., 2015). In the present pilot study, we used a standard DNA extraction protocol based upon the Human and Earth Microbiome Project protocols and compared preservation-free stool to that stored in RNAlater® (Gilbert et al., 2014). In addition, each stabilization condition was tested at multiple temperatures and lengths of time, to simulate those experienced in the mailing of samples through the Canadian postal service, which can range in extremes of temperature beyond 35 °C and −35 °C. The use of RNAlater® was selected as it is nontoxic and quickly stabilizes RNA and DNA, thus may be easily utilized by study participants during at-home stool collection. Based on previous literature, we hypothesized that the use of RNAlater® would contribute to an initial skew in the microbiota profile compared to immediately extracted, unpreserved stool samples, but would minimize alterations in the profile over storage at increased temperature (Flores et al., 2015; Hale et al., 2015; Liang et al., 2020; Voigt et al., 2015). We also speculated that unpreserved samples would alter greatly in their microbiome profile over increasing temperatures and days in storage, while frozen samples would most closely resemble the immediately extracted, unpreserved stool samples (Hang et al., 2014).

2.3 Materials and Methods

2.3.1 Stool sample collection and processing

Three volunteers were recruited at St. Joseph's Hospital in London, Canada. The Western University Health Science Research Ethics Board granted ethical approval for all experiments involved in the study. The methods were carried out in accordance with the approved guidelines and all participants provided written informed consent (REB #105443). The group was unrelated and comprised of one male recently immigrated to Canada from China, and one male and female Canadian of European ethnicity. Participants ranged in age from 23 to 29 years old and had not used antibiotics recently. Samples were collected in sterile containers from which 5 g was immediately removed (within 10 min of collection), suspended with 10 ml of sterile phosphate buffered saline (PBS), and vortexed in a sterile centrifuge tube until homogenization. A protocol was designed to model direct shipment of soiled toilet paper in sterile collection bags, as it would be a simple and more appealing method for study participants, compared to the use of sterile swabs. For this reason, 200 μL of the homogenized stool was aliquoted onto a sheet of clean toilet paper for every storage method condition to be tested (Figure 5). For samples stored in a preservation agent, the
paper was fully submerged in 5 mL RNaLater®, before being placed in a sterile 15 mL centrifuge tube; unpreserved paper was dry. A total of 18 aliquots were prepared from each individual (Figure 5, Table 2).

DNA extraction was performed in accordance with the Earth Microbiome Project standard protocols, with the MoBio PowerSoil® DNA Isolation Kit (Mobio, Carlsbad, CA). Sterile PBS (5 mL) was added to unpreserved samples immediately prior to DNA extraction. Samples in either RNaLater® or PBS were vortexed for 5 min prior to extraction to homogenize the toilet paper with the stool sample. The stool homogenate (250 μL) was added to the PowerBead tubes provided in the PowerSoil® kit. All samples were extracted in duplicate. The fresh extraction samples (N/A days in storage) were extracted immediately following the sample preparation, while the flash frozen samples were placed at −80 °C for 30 min, and then immediately extracted. DNA was stored at −20 °C following extraction.
Figure 5. Methodological schematic of study design
Table 2. Sample identifiers

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<td>Subject of origin</td>
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<td>Preservation method</td>
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For PCR amplification, 20 μL of the DNA template extract was transferred to 96-well PCR plates, in duplicate. These plates were then used with the BioMek® 3000 Laboratory Automation Workstation for automated PCR reagent set up. Amplifications of the V4 region of the 16S ribosomal RNA gene were carried out with the primers (5′-3′)

ACACTCTTTCCCTACACGACGCTTCCGATCTNNNNxxxxxxxGTGCC
AGCMGCCGCGGTAA and (5′-3′) CGGTCTCGGCTTCCTGCGACCTCTCTTCCGATCTNNNNxxxxxxxGGACTACHVGGGTWTCTAAT

wherein xxxxxxxx is a sample specific nucleotide barcode and the preceding sequence is a portion of the Illumina adapter sequence for library construction. Ten microlitres (3.2 pmol/μL) of a total of 32 primers (16 left and 16 right) with unique barcodes were arrayed in 96 well plates. The BioMek® robot transferred 2 μL of the DNA template into a plate containing 10 μL of each unique primer. Then 20 μL of Promega GoTaq® Colourless Master Mix (Promega, Maddison, WI) was added to the DNA template and primers. The final plate was firmly sealed with a foil PCR plate cover. This plate was placed in the Eppendorf Mastercycler® thermal cycler (Eppendorf, Mississauga, ON), where the lid was kept at 105 °C. An initial warm-up temperature of 95 °C was used for 2 min to activate the GoTaq®. Afterwards, the volumes underwent 25 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min. After completion, the temperature of the thermal cycler was held at 4 °C, and amplicons were then stored at −20 °C.

2.3.2 DNA sequencing and data analysis

Processing of DNA samples and DNA sequencing was conducted at the London Regional Genomics Centre at Robarts Research Institute (London, ON). Amplicons were quantified using pico green (Quant-It; Life Technologies, Burlington, ON) and pooled at equimolar concentrations before cleanup (QIAquick PCR clean up; Qiagen, Germantown, MD). The final samples were sequenced using the MiSeq by Illumina® platform, with 2 × 300 bp paired-end chemistry. The returned reads were then analyzed using Mothur, the SILVA database, Core R packages, and ALDEx2 (Fernandes et al, 2013, Quast et al., 2013; R Core Team, 2018; Schloss et al., 2009). Fastq files may be accessed in the NCBI Sequence Read Archive: BioProject ID PRJNA418287.
2.4 Results

After employing all quality control criteria described above, 234 OTUs were identified from the 216 samples (18 conditions × 3 individuals × 2 duplicate DNA extractions × 2 duplicate PCR reactions). OTUs were filtered and removed if the mean read count across all samples was less than 10. A bar plot and dendrogram illustrating the relative proportion of genera in the samples is shown in Figure 6. The dendrogram illustrates that samples strongly clustered by each participant when analyzed by Aitchison distance, an appropriate metric for compositional data (Gloor and Reid, 2016; Gloor et al., 2017; Van den Boogaart and Tolosana-Delgado, 2013). Figure 6 also illustrates that the three participants were dominated by the genera *Bacteroides*, *Faecalibacterium*, and *Prevotella*. Using a centre log ratio (CLR) transformation, the sample-wise Aitchison distances were generated, and principal components analysis performed (Figure 7). Components 1 and 2 explain 79.3% of total variance. The samples from each individual participant clustered together regardless of preservation method (Figure 7). However, when observing on a per-participant basis the samples did not cluster by any storage condition (data not shown). When summarized to phylum or genus level, no trends in taxonomy were associated with the samples between or within individuals (data not shown).

The distance between technical and inter-individual samples was compared (Figure 8). Paired Aitchison distances between PCR replicates, DNA extraction replicates, and preservative method (technical variants) were found to be lower than distances between paired samples from different participants. To further characterize the significance of the separation between individuals, k-means clustering was utilized to attempt partitioning all samples into n groups. When n = 3 which is the number of participants, samples clustered without overlap into groups based on the participant of origin (Figure 9). On a within-participant basis, no sample clustering was observed for any number n.

The centres of the clusters (n = 3) on the PCA (Figure 7) were determined and sample distance-to-centre calculated. The samples closest to centre may be considered the most reliable or “true” within the cluster. Table 3 displays the distances of the closest 10 samples within each cluster to the cluster centre. Within and between participants there is no pattern for storage methods that cluster most closely to the centre.
The average of all PCR and DNA extraction replicates of the fresh extracted samples was then calculated to determine a “fresh centre” for each subject. Table 4 displays the distance to fresh centre of the samples from which the average was generated, as well as the closest 10 samples within each cluster to the fresh centre; these are the 10 samples that most closely the fresh sample in microbial composition. For participants 1 and 3, samples that were stored at −80 °C without RNAlater most closely resembled the microbiota of fresh samples. For participant 2, there does not appear to be a definitive relationship between the storage method of the 10 closest samples to the fresh samples.
Figure 6. Microbial composition of stool samples

Bar plot displays the stool microbiota profiles. Each bar represents a single sample, with each colour representing a different bacterial genus. Samples are clustered hierarchically by the dendrogram, where samples originating from Subjects 1, 2, and 3 are coloured on red, green, and blue branches, respectively.
PCA was performed on CLR-transformed Aitchison distances from samples with pooled PCR and DNA extraction duplicates. Samples (points) that cluster together on the two-axis plot are similar in microbiota composition and abundance. As shown, samples cluster by participant of origin without overlap, but no distinct patterns exist between storage conditions. This exploratory analysis illustrates that 79.3% of the total variance of the samples is explained by the first two components.

Figure 7. Principal component analysis of all samples
Distance was measured between technical replicates and paired inter-individual samples. The violin plot illustrates the upper and lower quartiles with median and an overlapped kernel density estimation. The technical replicates were separated by significantly lower Aitchison distance than the paired inter-individual samples.

**Figure 8. Aitchison distance between paired experimental replicates**

Distance was measured between technical replicates and paired inter-individual samples. The violin plot illustrates the upper and lower quartiles with median and an overlapped kernel density estimation. The technical replicates were separated by significantly lower Aitchison distance than the paired inter-individual samples.
Figure 9. K-means clustering silhouette plot

When \( n = 3 \) groups, k-means clustering analysis successfully partitions all samples based on subject of origin. High silhouette coefficients (0.68-0.79) illustrate high confidence and large separation between sample clusters.
Table 3. Distance of the ten closest samples within each participant cluster to the cluster centroid

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Table 4. Distance of the 10 closest samples to the "fresh centre" within each participant cluster

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2.5 Discussion

This study investigated if and how sampling and storage would impact the microbial community structure of fecal samples collected on toilet paper. With the use of various techniques including principal components analysis and k-means clustering, the samples consistently grouped by participant, not storage method. In the principal component analysis, all samples clustered based on their participant of origin without overlap. Technical replicates of participant samples had significantly less variation than the variation between paired samples from different participants. Similar to other studies in healthy people, we showed that *Bacteroides*, *Faecalibacterium*, and *Prevotella* dominated the gut microbiotas of the three participants (Arumugam *et al*., 2011; King *et al*., 2019). Together, these results illustrate that regardless of the storage conditions, a participant's stool samples still resemble “self”.

It was somewhat surprising that no distinguishing patterns were found between storage methods and variance within participants (Tables 3 and 4). It was expected that the use of RNALater® would initially alter the microbiome of exposed samples, skewing it to appear less like the fresh sample (Choo *et al*., 2015; Gorzelak *et al*., 2015; Hale *et al*., 2015; Liang *et al*., 2020). Due to the preservative nature of RNALater®, it was expected that samples would be stabilized over storage in increased temperatures (Hale *et al*., 2015); surprisingly neither of these trends were observed. In two of the three participants, immediate freezing of the samples at −80 °C resulted in the microbiota most closely resembling that found in fresh stool. In the other participant, there were no storage methods that consistently resembled the fresh sample. These results imply that there is no “perfect” method of sample storage appropriate for every participant. It appears that utilizing a preservative agent such as RNALater® is unnecessary for samples stored up to seven days from −80 °C to 32 °C, with an added study cost and inconvenience for participants.

The analysis of next generation sequencing has become the favoured method to investigate the composition of the microbiome and how it relates to various clinical conditions. The methodology is sensitive and requires much standardization to achieve reproducible results. A strength of the current study is the use of paired-end Illumina
sequencing and exploration of the variation in DNA extraction and PCR technical replicates, which allowed us to explore the extent to which our entire sample-processing and sequencing pipeline may inadvertently vary between samples. Our results demonstrate that technical variation between sample replicates was extremely minor and did not contribute to demonstratable downstream effects when our bioinformatic filtering was applied. Because some of the methods utilized herein may be influenced by human error, others utilize robotic pipetting, and all were subject to basal variability inherent in Illumina MiSeq runs, this study was an important primary validation in establishing the microbiome analysis standard operating procedures at our centre (Wen et al., 2017).

This study also verifies the use of toilet paper sampling, which is an extremely convenient and low-cost methodology for acquiring fecal samples for the purpose of microbiome analysis in clinical studies. Although the method of aliquoting stool onto toilet paper used herein does not account for the transfer of skin microbes during at-home sampling, the potential contamination from skin microbes would be negligible in abundance (Sender et al., 2016).

The storage treatments investigated did not significantly impact the microbial communities and do not validate the use of storage preservative agents in the short term, although no conclusions can be made regarding the efficacy of such preservatives for long-term storage. These findings are comparable to similar studies in the literature (Dominianni et al., 2014; Nel van Zyl et al., 2020; Voigt et al., 2015). This method can be employed where patients are required to conduct at-home sampling, and based on the temperatures investigated herein, validate this method for the shipment of samples via standard postal service. Depending on the season, samples in post may be exposed to temperatures above 30 °C or below 0 °C for up to a week, however we found that even storage for 7 days at 32 °C did not push the microbial communities beyond the recognition of “self” when compared to fresh samples from the same participant.

Despite these conclusions, we suggest careful documentation of sample collection if this method is to be utilized in future clinical studies. For example, the study participant should record the date of sample collection, when it was mailed, and the lab should
record the samples receipt with regional weather trends throughout the sample’s shipment. Although no differences were determined based on temperature in this short-term pilot study, curated sample metadata should always be utilized in downstream analysis to identify the true source of microbial community variability (Allaband, et al., 2018). If this method is utilized in future studies, we also suggest the use of nuclease-free transport bags (such as VWR CA93000-724), such that external contamination after sample collection is minimized. Indeed, regardless of which sample collection method is chosen, consistency of methodology between samples in a study (down to the container) is an important feature of collection (Liang et al., 2020).

In conclusion, although immediate processing of samples is preferable, fecal samples collected on toilet paper can be stored and shipped short-term in varied temperatures without the preservative agent RNAlater®, without fear of vast microbial changes.

2.6 References


Chapter 3

3  The microbiome at multiple body sites in a kidney stone patient population: revisiting the role of Oxalobacter formigenes in stone formation

3.1  Abstract

Mounting evidence suggests a role for both the urinary and gut microbiome in nephrolithiasis. Intestinal colonization of Oxalobacter formigenes has been proposed to reduce the risk of oxalate stone disease through its capacity to degrade calcium oxalate. However, literature on the impact of this microbe in nephrolithiasis is controversial. The aim of the present study was to use a systems-level approach to fully characterize both the urinary and gut microbiota of stone formers and healthy controls and assess the bacterial contribution to this disease. Urine and fecal samples from 83 stone formers (SF) and 30 healthy controls (HC) were evaluated with 16S rRNA gene sequencing, and whole shotgun metagenomic sequencing. Stone fragments and intraoperative urine from SFs were also analyzed. The SF gut microbiota was significantly enriched in Enterobacteriaceae and potential uropathogens, however there was no observable difference in relative abundance of O. formigenes compared to HC. The urinary microbiota and oxalate concentrations were altered between HC and SF, and significantly differed in the SFs over time (pre-operative vs. intraoperative urine samples), exhibiting enrichment in pathogenic bacteria following perioperative antibiotic treatment. Interestingly, kidney stones harboured a microbiota distinct from urine, and this was not dictated by the stone’s crystalline composition. The gut microbiota of SFs was aberrant compared to HC in several functional capacities, including increased resistance to toxic compounds (antibiotics and heavy metals), altered micronutrient biosynthesis and utilization implicated in host oxalate homeostasis, and an elevated osmotic stress response. Together these findings suggest that the microbiota of SF is altered in a multimodal manner, aggravating stone formation. The microbiome as a system likely plays a much more significant role in nephrolithiasis beyond the dogmatic perspective that O. formigenes colonization is the key to stone prevention.
3.2 Introduction

Kidney stone disease, or nephrolithiasis, is an extremely prevalent condition that causes significant morbidity to sufferers and is a draining financial burden to public health (Pearle et al., 2005). Although classically believed to be an affliction of the obese and middle-aged white man, prevalence has risen in recent decades, specifically in young women in children (Scales Jr. et al., 2007; Scales Jr. et al., 2012; Soucie et al., 1994; Tasian et al., 2016). The human microbiota, defined as the microorganisms present in a certain environment, is known for its role in systemic human health and disease, including metabolic syndrome, cardiovascular disease, and diabetes (Gan et al., 2014; Gurung et al., 2020; Turnbaugh et al., 2006; Whiteside et al., 2015). Interestingly, these conditions are known comorbidities associated with nephrolithiasis, and their increasing prevalence over recent years along with that of stone disease indicate systemic declines in our population’s overall health (Logan et al., 2016). Importantly, the human gut microbiota is also implicated in nephrolithiasis, but a consensus on the mechanisms behind this relationship remain elusive.

Calcium oxalate (CaOx) is the most common crystalline composition of stones, followed by calcium phosphate, uric acid, struvite, and cystine (Moe, 2006). Oxalate is a toxic terminal metabolite produced endogenously and consumed in the diet (Hatch and Freel, 2005). The infamous Oxalobacter formigenes utilizes oxalate as its sole carbon source, and some studies have shown that people intestinally colonized by the bacterium have lower urinary oxalate levels and are subsequently at lower risk of developing CaOx stones (Kaufman et al., 2008; Jiang et al., 2011). However, many studies also find no difference in colonization rates between healthy persons and stone formers (Magwira et al., 2012; Miller et al., 2019; Tang et al., 2018; Ticinesi et al., 2018). Other members of the gut microbiota are capable of degrading oxalate including various lactobacilli and bifidobacteria (Miller and Dearing, 2013). In kidney stone patients, supplementation with oxalate-degrading bacteria has been suggested as a potential preventive therapy, however trials thus far have been limited and inconclusive (Hoppe et al., 2006; Siener et al., 2013; Klimesova et al., 2015). It remains unclear if direct oxalate metabolism by gut colonizers is the key to preventing kidney stones.
Beyond oxalate utilization, previous studies have demonstrated generalized “dysbiosis” in the intestinal microbiota of kidney stone formers (Miller et al., 2019; Suryavanshi et al., 2018; Stern et al., 2016; Tang et al., 2018; Ticinesi et al., 2018; Zampini et al., 2019). However, the significant perturbations determined in these studies are often not consistent; this may be an artifact of small sample size, or different sequencing and analysis methodologies. Most of the studies to date have also focussed primarily on the presence or absence of *O. formigenes* and direct oxalate utilization pathways, but the narrow focus towards these analyses may be overemphasizing their true functional significance to the disease pathology.

Although historically believed to be sterile, the urinary microbiota in healthy people has been well described in the last decade (Wolfe et al., 2012); it is compositionally distinct and of lower abundance than the microbiomes of the gut and vagina (Whiteside et al., 2015). The recent characterization of this microbiome has led investigators to question the role it may play in nephrolithiasis (Xie et al., 2020). Indeed, while struvite stones are known to be associated with urinary tract infections (UTI), recent culture-dependent and independent studies have confirmed the presence of bacteria in calcium-based stones (Barr-Beare et al., 2015; Dornbier et al., 2019; Zampini et al., 2019). As direct bacterial oxalate utilization is likely not at play in the urinary tract, it is unknown how these bacteria may be contributing to the disease, and whether these stone-bound microbes result from an aberrant urinary microbiota.

The use of multiple classes of oral antibiotics has been directly associated with increased risk of nephrolithiasis, with aggravated effects coinciding with more recent exposure and antibiotic use in younger ages (Tasian et al., 2018). The long-term perturbations to the microbiome caused by antibiotics and the associated stone risk are yet another indication of a role for the microbiome in this disease. Which body site is of most significance to the pathology is still unclear, and a role for the microbiome beyond direct oxalate utilization has been underexplored.

The aim of the present study was to fully characterize the urinary and gut microbiota of kidney stone formers and healthy controls to assess the bacterial contribution to
nephrolithiasis. It was hoped that this would provide insights to the bacterial implications for stone formation from multiple body sites and provide foundational knowledge upon which personalized medicine and targeted therapies could be developed for the prevention and treatment of nephrolithiasis.

3.3 Materials and Methods

3.3.1 Study design and sample collection

Nephrolithiasis patients were recruited from the Urology Department at St. Joseph’s Hospital in London, Ontario. Ethical approval for the study was granted by Lawson Health Research Institute (CRIC R 15-117) and the Health Sciences Research Ethics Board at the University of Western Ontario (REB #105443, Appendix B) in London, Ontario. Written consent was obtained from all the study participants at the time of study inclusion and the methods were carried out in accordance with the approved guidelines.

The study aimed to recruit up to 200 participants over a 5-year period, but the final numbers of subjects enrolled were 83 stone patients and 30 healthy controls. Inclusion and exclusion criteria for the participants are provided in Table 5. All stone patients who met the inclusion criteria were recruited during regularly scheduled clinic appointments, and healthy control subjects were approached in the community and through poster advertisements. Upon recruitment, subjects were asked about relevant demographic and medical history including antibiotic usage and their history of urinary tract infections. Following enrolment, participants provided a mid-stream urine sample, oral swab, and mailed in a fecal sample on toilet paper (Al et al., 2018). During surgical stone removal, additional clinical samples were collected: urine, upon first insertion of the catheter and prior to instilling saline into the urinary tract, and where possible, stone fragments. These were placed by the surgeon into a sterile collection cup. An operating room (OR) environmental control sample was also collected where a sterile urine container containing 200 uL of nuclease-free water (Ambion, Mississauga, ON, CAN) was left open beside the patient for the duration of their surgery.

From home, participants completed a diet history questionnaire either by paper copy which was mailed back to the laboratory, or online through the diet questionnaire
website. The food frequency questionnaire investigated past year intake and included questions about portion size, providing outputs with nutrient estimates (Subar et al., 2001).

3.3.2 Clinical sample processing

All processing of clinical samples was performed with PCR-grade filter pipette tips in a biosafety cabinet (FroggaBio, Toronto, ON, CAN). All urine samples (i.e. from healthy subjects as well as pre-operative and intra-operative urine from stone patients) were processed in two portions within 2 hours of collection. Where possible, 10 mL of whole urine was collected and frozen at -80 °C for high-performance liquid chromatography (HPLC) analyses of urinary metabolites. The remainder was stored for future 16S rRNA gene sequencing: the entire remaining volume of urine was centrifuged for 10 minutes at 5,000 x g, after which the supernatant was decanted off and the pellet was stored dry at -80 °C until DNA extraction. If the total urine volume was under 25 mL, only 2 mL of whole urine was reserved for HPLC. The urine volume that resulted in the pellet for 16S rRNA gene sequencing was recorded to identify confounding factors in the downstream sequencing analysis associated with processing conditions.

Oral swabs and fecal samples on toilet paper were frozen at -80 °C for future 16S rRNA gene sequencing: oral swabs within 2 hours of their initial collection and fecal samples within 2 hours of their receipt to the laboratory. Within 2 hours of collection, the water inside the OR-environmental control sample was shaken in the cup for 2 minutes and the entire volume was transferred to a PCR-grade Eppendorf tube (Thermo Scientific, Waltham, MA, USA), and frozen at -80 °C for future 16S rRNA gene sequencing.

Within 2 hours of their initial collection, one stone fragment per patient was transferred to a PCR-grade Eppendorf tube, frozen at -80 °C and stored until DNA extraction. If further fragments were available, they were cultured using the streamlined Extended Quantitative Urine Culture technique (EQUC) (Hilt et al., 2014; Price et al., 2016). Stones for culture were first surface sterilized with 70% ethanol for 10 seconds, followed by 2 washes with sterile phosphate buffered saline (PBS). They were then pulverized with a mortar and pestle sterilized with 70% ethanol until only sand-like fragments were
present. The fragments were suspended in 1000 µL of sterile PBS, and 100 µL of the suspension was pipetted and spread onto plates of Columbia blood agar (CBA, BD, Mississauga, ON, CAN) with 5% defibrinated sheep’s blood (Cedarlane, Burlington, ON, CAN), colistin-nalidixic acid agar (CNA, BD), MacConkey agar (BD), and CHROMagar™ orientation plates (CHROMagar, Paris, FR). The CBA and CNA plates were incubated at 5% CO₂ for 48 hours, and the MacConkey and CHROMagar plates were incubated aerobically for 48 hours. If colonies were cultured, DNA was extracted with the Monarch Genomic DNA Purification Kit (New England Biolabs, Whitby, ON, CAN) as per the manufacturer’s instructions. The DNA was then PCR amplified with pA/pH primers (8F and 1522R) of the 16S gene as previously described and Sanger sequenced at the London Regional Genomics Centre (http://www.lrgc.ca; London, ON, CAN) (Edwards et al., 1989). Sequences were then assessed with the Ribosomal Database Project to determine colony identification (https://rdp.cme.msu.edu).

3.3.3 High performance liquid chromatography

Urine samples were analyzed with HPLC to determine creatinine and oxalate levels (Maalouf et al., 2011; Murray et al., 1982). Reserved whole urine samples were thawed and vortexed for 30 seconds. Using PCR-grade filter tips, 50 µL of urine was transferred to an Eppendorf tube for creatinine quantification, and 950 µL of urine was transferred to a 15 mL conical tube for oxalate quantification.

For quantifying creatinine, 450 µL HPLC H₂O and 500 µL of HPLC acetonitrile were added to the urine and vortexed. The urine mixture was incubated at 4 °C for 15 minutes, facilitating precipitation of debris. Samples were then centrifuged for 15 minutes at 16,000 × g at 4 °C and filtered through 0.2 μm syringe filters into labelled amber HPLC vials (Agilent, Mississauga, ON, CAN). Standards of creatinine were prepared in HPLC H₂O at concentrations of 1 ppm, 5 ppm, 10 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm, and 300 ppm. The Agilent 1100 HPLC was utilized with the conditions stated in Table 6.

For oxalate quantification, the 950 µL of urine (or oxalic acid standards at concentrations of 1 ppm, 10 ppm, 25 ppm, 50 ppm, 100 ppm, 150 ppm, 200 ppm, 250 ppm) was
combined with 50 μL of 10 M HCl and 1 mL of 0.1 M o-phenylenediamine dissolved in 4 M HCl and vortexed. The tubes were capped, being careful to tighten the lids as much as possible. The tubes were then incubated in a laboratory oven at 100 °C for 6-7 hours, then moved to 4 °C overnight. The following day, volume in the tube was carefully inspected and tubes that had experienced cracking or evaporation were discarded, requiring repeat processing. Five hundred μL of 200 mM KHPO₄ (pH 7.0) and 480 μL of 10 M KOH were added to the tubes with gentle vortexing. One mL of the mix was then transferred to labelled Eppendorf tubes and incubated at 4 °C for 15 minutes, then centrifuged for 15 minutes at 16,000 × g at 4 °C. Large pellets were present and were carefully avoided when transferring the entire volume to a 0.2 μm syringe filters; samples were filtered into labelled amber HPLC vials (Agilent). The HPLC was utilized with the conditions stated in Table 6.

### 3.3.4 DNA extraction

On the day of DNA extraction, the samples were thawed and processed in a sterile biosafety hood. Using tweezers sterilized with RNase AWAY™ (Thermo Fisher Scientific, Waltham, MA, USA), the kidney stone was transferred to a sterile cell strainer mounted onto an empty 50 mL conical tube (Thermo Fisher Scientific). New sterile cell strainers and conical tubes were used for every sample. Two mL of nuclease free water was gently rinsed over the external surface of the stone. The stone was then transferred to a mortar and pestle that was sterilized with 5% sodium hypochlorite followed by RNase AWAY™ and pulverized into sand-like fragments. The fragments were suspended in 100 μL of nuclease-free water and pipetted directly into wells of the bead plate of the DNeasy PowerSoil HTP 96 Kit utilized for DNA extraction (Qiagen, Toronto, ON, CAN). Urine pellets were thawed and suspended in 100 uL of nuclease-free water, then pipetted into the bead plate. The 100 uL OR environmental control water sample was transferred directly to the bead plate. Toilet paper samples were dissected and trimmed with RNase AWAY™-treated scissors and forceps such that a piece of visibly soiled paper approximately 1 cm² in size was added directly to the bead plate.

Urine, stone, and OR environmental control samples were randomized across DNA extraction plates together; fecal samples were extracted on separate plates to mitigate
potential contamination to the other samples which were of significantly lower biomass in comparison.

Two wells in every DNA extraction plate were left empty and acted as negative controls. Two positive controls, or “spikes”, were added to each plate and were 100 μL of pure bacterial culture: Spike 1 was *Escherichia coli* strain DH5α, and Spike 2 was *Staphylococcus aureus* strain Newman. For preparation of the spikes, a single colony of the bacteria was inoculated into 10 mL of Luria-Bertani (LB) broth and grown overnight at 37 °C. One hundred 100 μL aliquots of the overnight cultures were portioned into 1.5 mL Eppendorf tubes and frozen at -80 °C. For each DNA extraction plate, a single tube of both spikes was thawed and pipetted directly into the PowerSoil HTP bead plate with PCR-grade filter tips.

DNA was isolated from samples using the DNeasy PowerSoil HTP 96 Kit according to the manufacturer’s instructions. Briefly, 750 μL of bead solution and 60 μL of Solution C1 were added to the bead plate loaded with all samples and controls. Plates were shaken for 20 minutes at speed 20 using the MoBio 96-well plate shaker (Qiagen), then centrifuged for 10 minutes (all centrifuge steps were conducted at room temperature at 2250 x g). Five hundred μL of the supernatant was added to a fresh plate with 250 μL Solution C2 and mixed by pipetting. The plates were incubated at 4 °C for 10 minutes followed by centrifugation for 10-minutes. The resulting supernatant was then transferred to a fresh plate and the centrifugation step was repeated. Approximately 600 μL of the solution was then transferred to a fresh plate containing 200 μL Solution C3 and mixed by pipetting. The plate was incubated at 4 °C for 10 minutes followed by centrifugation for 10-minutes. The entire volume, with the exception of the pellet, was again transferred to a fresh plate for centrifugation. Carefully avoiding the loose residual pellet, 650 μL was transferred from each well to a fresh plate containing 1300 μL Solution C4. The plates were then sealed with sealing tape and stored at 4°C overnight. The following day the plates were briefly centrifuged and sealing tape was removed. Solution in the wells was then mixed by pipetting and 500 μL was transferred to a Spin Plate, which was centrifuged for 5 minutes. The flow-through was discarded, and this step was repeated until the entire sample-Solution C4 mix was processed through the Spin Plate. A volume
of 500 μL Solution C5-D was then added to the Spin Plate and centrifuged twice for ten minutes, where the flow-through was discarded between spins. 100 μL Solution C6 was then added to the Spin Plate, incubated at room temperature in the biosafety hood for 10 minutes and the resulting DNA was eluted to a Microplate via centrifugation for 15 minutes. DNA was stored at -20 °C until downstream processing.

3.3.5 16S rRNA gene sequencing

Urine, stone, and OR-environmental control samples underwent 16S rRNA gene sequencing. PCR amplification was completed using the Earth Microbiome universal primers (515F and 806R) which are specific for the V4 variable region of the 16S rRNA gene (Figure 4). Primers contained an Illumina adapter, followed by four random nucleotides, one of 24 unique 12-mer barcodes, and the annealing left or right primer (Figure 4) (Parada et al., 2016). Primers and barcode sequences are listed in Table 7. PCR reagent set-up was performed using a Biomek® 3000 Laboratory Automation Workstation (Beckman-Coulter, Mississauga, ON, CAN). Ten μL of each left- and right-barcoded primers (3.2 pMole/μL) were arrayed in 96-well plates (Axygen-Corning, Oneota, NY, USA) such that each well contained a unique combination of left- and right-barcodes (up to a maximum of 576 unique combinations). Two μL of DNA template was added to the primer plate, followed by 20 μL of Promega GoTaq hot-start colourless master mix (Promega, Madison WI, USA). The reaction was briefly mixed by pipetting, then plates were sealed with foil plate covers (Axygen-Corning) and centrifuged for 2 minutes at room temperature at 2250 x g.

Amplification was carried out using an Eppendorf thermal cycler (Eppendorf, Mississauga, ON, CAN), where the lid temperature was maintained at 104 °C. An initial warm-up of 95 °C for 4 minutes was utilized to activate the GoTaq, followed by 25 cycles of 1 minute each of 95 °C, 52 °C, and 72 °C.

DNA extraction was completed across a total of 3 x 96-well plates. Sequencing was carried out at the London Regional Genomics Centre. Amplicons were quantified using pico green (Quant-It; Life Technologies, Burlington, ON) and pooled at equimolar concentrations before cleanup (QIAquick PCR clean up; Qiagen, Germantown, MD).
Using the 600-cycle MiSeq Reagent Kit (Illumina Inc., San Diego, CA, USA), paired-end sequencing was carried out as $2 \times 260$ cycles with the addition of 5% $\Phi$X-174 at a cluster density of $\sim 1100$. Data was exported as raw fastq files and analyzed using R, DADA2, the SILVA database (version 132), and ALDEx2 (Callahan et al., 2016; Fernandez et al., 2013; Quast et al., 2013; R Core Team, 2019).

3.3.6 Shotgun metagenomic sequencing

Fecal samples from 25 healthy control participants and 36 confirmed calcium-oxalate stone forming patients underwent shotgun metagenomic sequencing at The Centre for Applied Genomics (TCAG) at The Hospital for Sick Children in Toronto, ON, CAN. DNA concentration was quantified using the Qubit™ dsDNA HS Assay Kit (Invitrogen, Thermo Fisher Scientific). Approximately 100 ng of DNA was PCR amplified and prepared for sequencing using the Nextera DNA Flex Library Prep Kit (Illumina, Inc.). The amplified library was purified and enriched for amplicons $\sim 350$ bp, then sequenced using an S1 Flowcell on the Illumina NovaSeq 6000. Reads were exported as fastq files, quality assessed using FastQC (Andrews, 2010), trimmed with Trimmomatic (Bolger et al., 2014), and mapped against the human genome (Hg38) using Bowtie2 (Langmead and Salzberg, 2012). Human reads were discarded, and the remaining unmapped reads were utilized in downstream analyses. Taxonomy was assigned with Kaiju (Menzel et al., 2016) and reads were assembled into contigs using MetaSPAdes (Nurk et al., 2017). Functional annotation was determined based on alignment to the SEED database (Overbeek et al., 2014). Downstream statistical analysis was performed with core R packages, as well as Aldex2, vegan, MaAsLin2, and LEfSe (Fernandez et al., 2013; Morgan et al., 2012; Oksanen et al., 2019; R Core Team, 2019; Segata et al., 2011).
Table 5. Study participant inclusion and exclusion criteria

<table>
<thead>
<tr>
<th>Kidney stone patients</th>
<th>Inclusion</th>
<th>Exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>At least 18 years of age</td>
<td>Taking/taken any antibiotic during the previous 30 days</td>
<td></td>
</tr>
<tr>
<td>Scheduled for PCNL or URS as treatment of urinary stone disease</td>
<td>Has an active gastro-intestinal infection</td>
<td></td>
</tr>
<tr>
<td>Able and willing to provide informed consent</td>
<td>Previously enrolled in this trial</td>
<td></td>
</tr>
<tr>
<td>Able and willing to provide urine, saliva, and stool samples</td>
<td>In the opinion of the treating urologist, it is not in the patient’s best interest to participate</td>
<td></td>
</tr>
<tr>
<td>Able and willing to complete diet questionnaire at home</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Healthy control participants</th>
<th>Inclusion</th>
<th>Exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>At least 18 years of age</td>
<td>Taking/taken any antibiotic during the previous 30 days</td>
<td></td>
</tr>
<tr>
<td>Able and willing to provide informed consent</td>
<td>Has an active gastro-intestinal infection</td>
<td></td>
</tr>
<tr>
<td>Able and willing to provide urine, saliva, and stool samples</td>
<td>Has Crohn’s disease</td>
<td></td>
</tr>
<tr>
<td>Able and willing to complete diet questionnaire at home</td>
<td>Has ulcerative colitis</td>
<td></td>
</tr>
<tr>
<td>Undergo renal ultrasound to confirm stone-free status</td>
<td>Has had gastric bypass surgery</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Has a history of urinary stone disease</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Has a history of urosepsis in the past year (365 days)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Has a urinary diversion</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Has an indwelling catheter</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Performs clean intermittent catheterization</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Has previously enrolled in this trial</td>
<td></td>
</tr>
<tr>
<td></td>
<td>In the opinion of the treating urologist, it is not in the subject’s best interest to participate</td>
<td></td>
</tr>
</tbody>
</table>
### Table 6. HPLC running conditions

<table>
<thead>
<tr>
<th></th>
<th>Creatinine quantification</th>
<th>Oxalate quantification</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flow rate</strong></td>
<td>1 mL/minute</td>
<td>1 mL/minute</td>
</tr>
<tr>
<td><strong>Mobile phase</strong></td>
<td>60% ACN / 40% 5 mM KHPO$_4$ (pH 2.8)</td>
<td>15% ACN / 85% 50 mM KHPO$_4$ (pH 7.0)</td>
</tr>
<tr>
<td><strong>Column</strong></td>
<td>Poroshel 120 HILIC (4.6 × 150 mm, 4μm) – ambient temperature (Agilent)</td>
<td>Poroshel 120 EC-C18 (4.6 × 150 mm, 4μm) – ambient temperature (Agilent)</td>
</tr>
<tr>
<td><strong>Injection volume</strong></td>
<td>5 μL</td>
<td>5 μL</td>
</tr>
<tr>
<td><strong>Detector</strong></td>
<td>215, 4 (ref = 320, 120)</td>
<td>312.4 (ref = 355, 50)</td>
</tr>
<tr>
<td><strong>Retention time</strong></td>
<td>~2.2 minutes</td>
<td>~2.7 minutes</td>
</tr>
<tr>
<td><strong>Run time</strong></td>
<td>4 minutes</td>
<td>4 minutes</td>
</tr>
<tr>
<td>Name</td>
<td>Sequence (5’ – 3’)</td>
<td></td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Left Illumina adapter</td>
<td>ACACTCTTTCCCTACACGACGCTCTTCCGATCT</td>
<td></td>
</tr>
<tr>
<td>Right Illumina adapter</td>
<td>CGGTCTCGGCAATTCTCTGCTGACCACGCTCTTCCGATCT</td>
<td></td>
</tr>
<tr>
<td>Left primer (515F)</td>
<td>GTGCCAGCMGCCGCGGTAA</td>
<td></td>
</tr>
<tr>
<td>Right primer (806R)</td>
<td>GGACTACHVGGGTWTCTAAT</td>
<td></td>
</tr>
<tr>
<td>Barcode #1</td>
<td>TGCATACACTGG</td>
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</tr>
<tr>
<td>Barcode #2</td>
<td>ACTCACAGGAAT</td>
<td></td>
</tr>
<tr>
<td>Barcode #3</td>
<td>GTAGGTGCTTAC</td>
<td></td>
</tr>
<tr>
<td>Barcode #4</td>
<td>CAGTCGTTAAGA</td>
<td></td>
</tr>
<tr>
<td>Barcode #5</td>
<td>CACTACGCTAGA</td>
<td></td>
</tr>
<tr>
<td>Barcode #6</td>
<td>GCTCGAAGATTTC</td>
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</tr>
<tr>
<td>Barcode #7</td>
<td>TGAACGTTGGAT</td>
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</tr>
<tr>
<td>Barcode #8</td>
<td>ATGGTTTACCCCG</td>
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</tr>
<tr>
<td>Barcode #9</td>
<td>CGAGGGAAAAGTC</td>
<td></td>
</tr>
<tr>
<td>Barcode #10</td>
<td>TACTACGTGGGC</td>
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</tr>
<tr>
<td>Barcode #11</td>
<td>GTTCCTCCATTAA</td>
<td></td>
</tr>
<tr>
<td>Barcode #12</td>
<td>ACGATATGGTCA</td>
<td></td>
</tr>
<tr>
<td>Barcode #13</td>
<td>TATCGACACAAG</td>
<td></td>
</tr>
<tr>
<td>Barcode #14</td>
<td>AGCATGTCACGTC</td>
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</tr>
<tr>
<td>Barcode #15</td>
<td>CCAGATATAGCA</td>
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<tr>
<td>Barcode #16</td>
<td>GTGTCCGGATTC</td>
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</tr>
<tr>
<td>Barcode #17</td>
<td>ATCGCACAGTAA</td>
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<tr>
<td>Barcode #18</td>
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<td>Barcode #19</td>
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<tr>
<td>Barcode #20</td>
<td>TGTAGGTGTGCT</td>
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</tr>
<tr>
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<tr>
<td>Barcode #22</td>
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<tr>
<td>Barcode #23</td>
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<tr>
<td>Barcode #24</td>
<td>TCGGTACGCTAC</td>
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</tr>
</tbody>
</table>
3.4 Results

3.4.1 Study recruitment and participant demographics

Participant recruitment began in August of 2015 and was completed in January of 2019 after the inclusion of 30 healthy control (HC) participants and 83 stone-forming (SF) patients. This yielded 178 urine samples, 113 oral swabs, 36 environmental (OR) control samples, 47 stone fragments, and 102 fecal samples. Healthy controls were matched to stone patients on the basis of age, BMI, and comorbidities, but differed in their smoking status and antibiotic history (Table 8). The stone patients were evenly divided between those that received percutaneous (PCNL) compared to ureteroscopic (URS) surgery.

3.4.2 Urinary oxalate quantification

The urinary oxalate concentration was measured in ppm with HPLC and normalized to creatinine concentration. The urinary oxalate/creatinine ratio was significantly higher in the SF patients at the pre-operative time point compared to both healthy controls and SF at the OR timepoint (Figure 10).

3.4.3 Diet history analysis

HC and SF participants did not significantly differ in their estimated intake of any of the 216 measured features from the diet history questionnaire after multiple testing correction with Bonferroni adjustment. Similarly, no differences were statistically significant between study participants when they were grouped by their history of stones (i.e. no history of urolithiasis versus recurrent stone formers). Dietary metrics of interest that may play a role in stone formation (including water consumption, caffeine, Vitamins B₆, C, D, and K, calcium, sodium, potassium, and oxalate) are displayed in Figure 11.
Table 8. Demographic and clinical data of study participants

<table>
<thead>
<tr>
<th>Metric</th>
<th>Healthy controls</th>
<th>Stone patients</th>
<th>$P$ value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No. enrolled</strong></td>
<td>30</td>
<td>83</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>11 (37)</td>
<td>32 (39)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>19 (63)</td>
<td>51 (61)</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>M (SD)</td>
<td>M (SD)</td>
<td></td>
</tr>
<tr>
<td>55 (11.25)</td>
<td>58 (11.5)</td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td><strong>BMI</strong></td>
<td>M (SD)</td>
<td>M (SD)</td>
<td></td>
</tr>
<tr>
<td>27.6 (7.1)</td>
<td>30.2 (5.0)</td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Other health features</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>History of stones</td>
<td>n (%)</td>
<td>n (%)</td>
<td></td>
</tr>
<tr>
<td>0 (0)</td>
<td>70 (84)</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>History of UTI</td>
<td>9 (30)</td>
<td>43 (51)</td>
<td>0.06</td>
</tr>
<tr>
<td><strong>Smoking status</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current smoker</td>
<td>0 (0)</td>
<td>12 (14.8)</td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>30 (100)</td>
<td>71 (85.7)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Comorbidities</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiac</td>
<td>3 (10)</td>
<td>8 (9.5)</td>
<td>0.99</td>
</tr>
<tr>
<td>Hypertension</td>
<td>8 (27)</td>
<td>32 (38)</td>
<td>0.37</td>
</tr>
<tr>
<td>Diabetes</td>
<td>3 (10)</td>
<td>16 (19)</td>
<td>0.39</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>3 (10)</td>
<td>9 (10.7)</td>
<td>0.99</td>
</tr>
<tr>
<td><strong>Years since antibiotic use</strong></td>
<td>M (SD)</td>
<td>M (SD)</td>
<td></td>
</tr>
<tr>
<td>5.53 (4.0)</td>
<td>3.5 (6.7)</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Stone removal procedure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PCNL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 (48.2)</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ureteroscopy</td>
<td>43 (51.8)</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>
Urinary oxalate concentrations [ppm] were determined with HPLC and normalized to creatinine levels [ppm]. SF Pre-Op urine had the highest oxalate concentrations (Kruskall-Wallis test with Dunn’s multiple comparisons). HC = healthy control participants (n = 29); Pre-Op = SF urine at preoperative timepoint (n = 83); OR = SF intraoperative urine (n = 55). * P < 0.05, ** P < 0.01.

Figure 10. Urinary oxalate levels are higher in stone formers than healthy controls
Approximate daily values of macronutrients of interest were compared between patient groups; no macronutrients were significantly different by two-tailed Mann Whitney test. HC = healthy control participants (n = 14); SF = stone former (n = 64); NHS = no history of stones (n = 22); RSF = recurrent stone forming patients (n = 56).
3.4.4 16S rRNA gene sequencing

Microbiota sequencing was performed on urine, stone, and OR control samples with the Illumina MiSeq platform. There was a total of 277 samples that yielded 14,989,061 reads, ranging from 148 to 774,051 reads per sample. An average of 19.58% reads were removed from each sample following quality filtration performed utilizing the DADA2 pipeline (Callahan, *et al.*, 2017). The remaining filtered reads (13,766,536) were assigned taxonomy with the SILVA (v132) training set. Samples and sequence variants (SVs) were then further pruned such that the final dataset utilized in all downstream analyses retained samples with greater than 1,000 reads, SVs present at greater than 1% abundance in any sample, and SVs with greater than 10,000 total reads across all samples. This cleaning reduced the dimensions of the dataset from 935 SVs and 277 samples down to 83 SVs and 232 samples. Two additional SVs were removed due to their alignment to human mitochondrial sequences. Due to the low-abundance nature of the samples, additional stringent assessment using the decontam R package was performed to assess the presence of likely contaminant sequence variants (Davis *et al.*, 2018). After assessing prevalence, frequency, and combined metrics of contaminant detection with decontam, one additional SV was determined to be a potential contaminant and removed from downstream analyses (Figure 12).

The most proportionally abundant genera in urine and stone samples were *Escherichia* (29.7%), *Lactobacillus* (12.8%), *Staphylococcus* (10.3%), *Gardnerella* (7.3%), and *Streptococcus* (7.3%). The sequence counts were centre log ratio (CLR) transformed, generating sample-wise Aitchison distances, which were analyzed with principal components analysis (PCA) (Gloor *et al.*, 2017). The PCA biplot displays modest clustering of samples based on sample type (Figure 13A). Sample types also differed by alpha diversity, whereby SF urine taken during stone-removal surgery (OR urine) had the lowest diversity, and the HC and pre-operative SF urine samples had the highest diversity (Figure 13B).

Significant confounders of microbiota variation were determined using the *envfit* function within the R package ‘vegan’ for various sample group comparisons (Table 9). General linear models (GLM) were then utilized in MaAsLin2 to further determine significant
effectors of microbial community variation whilst accounting for the confounders. With this technique, the relative abundance of several genera was determined to be discordant when evaluating the three urine sample types (HC, SF pre-operative, and SF-OR) (Figure 14A). Within stone patients, *Lactobacillus* and *Prevotella* spp. were relatively more abundant at the pre-operative time point, and *Acinetobacter*, *Escherichia*, and *Gardnerella* spp. were more abundant in the OR (Figure 14 B-F). Notably, *Gardnerella* spp. were relatively more abundant in HC compared to SF pre-operative samples (Figure 14G).

Samples from the SF cohort were determined to differ by sample type (Table 9) and several genera were determined to be significantly discordant using a GLM (accounting for relevant confounders) when comparing the urine and stone samples (Figure 15A). Stones had relatively more *Gardnerella* spp. and less *Acinetobacter* spp. compared to SF-OR urine (Figure 15B-C). Stones had relatively more *Atopobium*, *Gardnerella*, and *Staphylococcus* spp. and relatively less *Dialister*, *Lactobacillus*, and *Prevotella* than SF Pre-Op urine (Figure 15D-I).

Finally, stone samples were investigated to determine if microbial communities differed by stone composition. The time since participants’ most recent UTI was determined to be a confounder of stone microbiota (Table 9), however when this was accounted for in a GLM, there were no significant differences in stone microbiota based on stone composition (Figure 16). Several stone samples were also cultured utilizing the EQUC method as a proof of principle that the microbiota composition determined with 16S was indeed due to living, culturable bacteria present within the stone. Table 10 illustrates that several colonies were grown from stone fragments and largely correlated with the 16S rRNA gene sequencing findings.
SVs were assayed with decontam “prevalence”, “frequency”, and “combined” methods. SVs determined to be contaminants are blue and “true” SVs are pink. One SV (circled in black) was detected in all three methods and subsequently removed from the downstream analyses.

**Figure 12. Determination of contaminant sequence variants**

SVs were assayed with decontam “prevalence”, “frequency”, and “combined” methods. SVs determined to be contaminants are blue and “true” SVs are pink. One SV (circled in black) was detected in all three methods and subsequently removed from the downstream analyses.
A) PCA was performed on CLR-transformed Aitchison distances of all urine and stone samples. Each coloured point represents a sample. Distance between samples on the plot represents differences in microbial community composition, with 17.6% of total variance being explained by the first two components shown. Strength and association for genera are depicted by the length and direction of the gray arrows, respectively. Points are coloured by sample type and ellipses represent the 95% confidence intervals of sample types. B) Shannon’s Index of alpha diversity was compared between sample groups. OR urine samples from stone patients had the lowest diversity (Kruskall-Wallis test with Dunn’s multiple comparisons, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Figure 13. Compositional analysis of all urine and stone samples
Table 9. Significant covariates of microbiota variation at genus level PCA ordination

<table>
<thead>
<tr>
<th>Samples tested</th>
<th>Metadata Feature</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HC and SF Pre-Op urine</td>
<td>Time since most recent UTI (years)</td>
<td>0.04995</td>
</tr>
<tr>
<td>SF Pre-Op and SF-OR urine</td>
<td>Time of sampling (Pre-Op vs. OR)</td>
<td>0.00099</td>
</tr>
<tr>
<td>SF Pre-Op urine and Stone</td>
<td>Sample type</td>
<td>0.00999</td>
</tr>
<tr>
<td>SF- OR urine and Stone</td>
<td>Sample type</td>
<td>0.00099</td>
</tr>
<tr>
<td></td>
<td>PCNL or URS</td>
<td>0.03196</td>
</tr>
<tr>
<td></td>
<td>History of orthopedic conditions</td>
<td>0.00999</td>
</tr>
<tr>
<td>Stone samples</td>
<td>Time since most recent UTI (years)</td>
<td>0.04895</td>
</tr>
</tbody>
</table>

* P values calculated using the `envfit` function within the vegan R package
Relative abundance

Acinetobacter****

Escherichia*

Gardnerella*

Lactobacillus***

Prevotella**

Gardnerella*

Urine Samples

SF-PreOp

SF-OR

Healthy control

PC1: 9.6%

PC2: 8.8%
Figure 14. Compositional differences in urine samples

A) PCA was performed on CLR-transformed Aitchison distances of all urine samples. Each coloured point represents a sample. Distance between samples on the plot represents differences in microbial community composition, with 18.4% of total variance being explained by the first two components shown. Strength and association for genera are depicted by the length and direction of the gray arrows, respectively. Points are coloured by sample type and ellipses represent the 95% confidence intervals of sample types. B-F) Significantly altered genera between stone formers’ preoperative (PreOp) and OR urine, and G) stone formers preoperative (PreOp) and healthy control (HC) urine samples, as determined with general linear models accounting for significant covariates. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. 

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. 

...
Figure 15. Compositional differences in stone-former samples

A) PCA was performed on CLR-transformed Aitchison distances of all urine samples. Each coloured point represents a sample. Distance between samples on the plot represents differences in microbial community composition, with 18.2% of total variance being explained by the first two components shown. Strength and association for genera are depicted by the length and direction of the gray arrows, respectively. Points are coloured by sample type and ellipses represent the 95% confidence intervals of sample types. B-C) Significantly altered genera between stone formers’ OR urine and stones, and D-I) stone formers preoperative (PreOp) and stone samples, as determined with general linear models accounting for significant covariates. CLR-transformed relative abundances are plotted. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
PCA was performed on CLR-transformed Aitchison distances of all stone samples. Each coloured point represents a sample. Distance between samples on the plot represents differences in microbial community composition, with 36.1% of total variance being explained by the first two components shown. Strength and association for genera are depicted by the length and direction of the gray arrows, respectively. Samples are coloured by stone composition (major component shown for stones with mixed composition).

**Figure 16. Kidney stone microbiota is not dictated by stone composition**
<table>
<thead>
<tr>
<th>Participant ID</th>
<th>Culture +/-</th>
<th>Stone composition</th>
<th>Colony characteristics: 16S ID*</th>
<th>Dominant genus by 16S**</th>
</tr>
</thead>
<tbody>
<tr>
<td>171</td>
<td>+</td>
<td>Calcium oxalate</td>
<td>Yeast (not sequenced)</td>
<td>N/A, below filter cut-off</td>
</tr>
<tr>
<td>175</td>
<td>+</td>
<td>Struvite</td>
<td>Short Gram negative rods: <em>Proteus mirabilis</em></td>
<td><em>Proteus</em></td>
</tr>
<tr>
<td>177</td>
<td>+</td>
<td>Uric acid</td>
<td>Gram negative rods: <em>Escherichia coli</em></td>
<td><em>Escherichia</em></td>
</tr>
<tr>
<td>178</td>
<td>+</td>
<td>Calcium oxalate</td>
<td>Gram negative rods (not sequenced)</td>
<td><em>Gardnerella</em></td>
</tr>
<tr>
<td>181</td>
<td>-</td>
<td>Calcium oxalate</td>
<td>N/A</td>
<td>N/A, below filter cut-off</td>
</tr>
<tr>
<td>182</td>
<td>+</td>
<td>Calcium oxalate</td>
<td>Gram positive cocci and Gram negative rods (not sequenced)</td>
<td><em>Staphylococcus</em> and <em>Klebsiella</em></td>
</tr>
</tbody>
</table>

* Generic 16S colony identification with pA-pH primers
** 16S rRNA gene microbiota sequencing
3.4.5 Whole shotgun metagenomic sequencing

One hundred and two fecal samples were collected from which DNA was extracted. Of these, 61 were selected to be sequenced with whole shotgun metagenomics. These comprised samples from 25 healthy controls, and 36 confirmed calcium oxalate (CaOx) stone forming patients, all with high enough DNA yield (100 ng of ds DNA) and quality (260/280 between 1.8-2.0) for sequencing library preparation. After quality filtering and discarding reads that mapped to the human genome, samples contained an average of 21,805,652 reads from 4342 taxa (including bacterial, archaeal, and viral annotations). After aligning filtered reads to functional outputs within SEED, 9299 gene products were annotated (Overbeek et al., 2014). The total proportional abundance of unclassified reads ranged from 5.0% to 24.8% (mean 11.7%). The most abundant taxa were Bacteroides, Clostridium, Alistipes, Faecalibacterium, and Prevotella. The 100 most abundant taxa are shown in Figure 17, where each sample (column) is clustered by a hierarchical dendrogram based on Aitchison distance. With this method, samples that are more similar in composition are closer together on the tree; branches of the dendrogram were coloured based on cohort, but samples did not group by HC (purple) or SF (orange).

Based on the findings that samples did not group based on cohort, an investigation was performed to determine what participant factors explained the microbiota variability. Significant confounders were determined using the envfit function within the R package ‘vegan’ on all the gut metagenome samples from both taxonomic and functional annotations (Table 11). This pointed to samples being segregated by participant age as well as whether or not they were recurrent stone formers. Of all the patients in the SF cohort, nine were first time stone formers with no previous history of urolithiasis. Due to the findings that stone recurrence was a large driver of microbiota variation, further downstream analyses considered patients based on this feature, rather than just whether they were originally classified as HC or SF. Of note, dietary intake was not significantly different between participants with no history of stones (NHS) and recurrent stone formers (RSF) (Figure 11).

Alpha diversity of the gut samples was determined with Shannon’s Diversity Index based on taxonomic annotation. Notably, SF samples were not significantly different from HC, but participants with NHS had significantly higher diversity than RSF (Figure 18).
General linear models (GLM) were then utilized in MaAsLin2 to further determine significant effectors of microbiome variation whilst accounting for the confounders (Table 11). With this technique, the relative abundance of several genera and functional pathways was determined to be discordant between participants on the basis of stone history. Linear discriminant analysis was performed with LEfSe (Segata et al., 2011), and features with an effect size greater than 2 that also achieved a q value (FDR corrected P value) in MaAsLin2 of <0.05 were retained (Morgan et al., 2012).

Recurrent stone formers were found to have a higher relative abundance of several genera within the family Enterobacteriaceae, including Escherichia, Klebsiella, Shigella, and Citrobacter, among other taxa, compared to those with no history of stones (Figure 19). In contrast, study participants with no history of stones had higher relative abundance of Prevotella, Faecalibacterium, Roseburia, and Clostridium, among other taxa, compared to recurrent stone formers (Figure 19).

Recurrent stone formers were elevated in the relative abundance of many functional pathways of interest, including resistance to antibiotics and toxic compounds, organic sulfur assimilation, osmotic stress, and quinone cofactors (Figure 20). In contrast, study participants with no history of stones had higher relative abundance in general cellular metabolism pathways including protein biosynthesis, DNA repair, and transcription, amongst others (Figure 20). Specific proteins of interest with differential relative gene abundance between the groups are illustrated in Figure 21. Recurrent stone formers had increased relative abundance of genes associated with heavy metal resistance, antibiotic and antiseptic resistance, osmoprotectants, and L-ascorbate utilization, and lower relative abundance of pyridoxine metabolism and synthesis relative to participants with no history of stones (Figure 21). Interestingly, no differences in relative abundance of Oxalobacter formigenes (Figure 22) were detected between groups. Enzymes involved in oxalate degradation such as formyl-CoA transferase, oxalyl-CoA decarboxylase and oxalate decarboxylase were not annotated with SEED, however analysis is ongoing with a custom metagenomic database to annotate the relative abundance of these gene pathways between groups (Abratt and Reid, 2010; NCBI Resource Coordinators, 2018; Ticinesi et al., 2018).
Figure 17. Bacterial composition of healthy and stone former gut microbiome

CLR-transformed values of the 100 genera with highest average relative abundance are displayed. Samples (columns) are clustered according to average linkage clustering of per-sample Aitchison distance, and branches of the resulting dendrogram are coloured by participant cohort. Phylum and genus (or otherwise lowest available taxonomic assignment) are listed.
Table 11. Significant covariates of gut microbiome variation

<table>
<thead>
<tr>
<th>PCA ordination level</th>
<th>Metadata Feature</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Taxonomic mapping of metagenomic reads</strong></td>
<td></td>
</tr>
<tr>
<td>Genus</td>
<td>Age</td>
<td>0.007</td>
</tr>
<tr>
<td>Genus</td>
<td>History of stones</td>
<td>0.007</td>
</tr>
<tr>
<td>Genus</td>
<td>Approximate number of previous stones</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td><strong>Functional mapping of metagenomic reads</strong></td>
<td></td>
</tr>
<tr>
<td>SEED subsystem1</td>
<td>History of stones</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>History of surgical treatment of stone disease</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Approximate number of previous stones</td>
<td>0.002</td>
</tr>
<tr>
<td>SEED subsystem2</td>
<td>Approximate number of previous stones</td>
<td>0.008</td>
</tr>
<tr>
<td>SEED subsystem3</td>
<td>Approximate number of previous stones</td>
<td>0.007</td>
</tr>
<tr>
<td>SEED subsystem4</td>
<td>Approximate number of previous stones</td>
<td>0.003</td>
</tr>
</tbody>
</table>

* P values were calculated using the `envfit` function within the vegan R package on all gut metagenome samples
Shannon’s index of alpha diversity was compared between cohorts and between patients based on their history of stone formation: those with a history of stones had significantly lower intestinal diversity than those without a history of stone formation (Two tailed Mann-Whitney U test, \( P = 0.016 \)).

Figure 18. Microbial diversity is different between participants with and without a history of stones, rather than between cohorts
Bacterial genera (or otherwise lowest available taxonomic assignment) with significantly altered relative abundance between stone history groups. Taxa with LDA effect size greater than 2 are plotted and coloured based on patient group of enrichment.

**Figure 19. Differential genera between stone history groups**

Bacterial genera (or otherwise lowest available taxonomic assignment) with significantly altered relative abundance between stone history groups. Taxa with LDA effect size greater than 2 are plotted and coloured based on patient group of enrichment.
Higher relative abundance in participants with:
- No history of stones
- Previous stones

Figure 20. Differential functional pathways between stone history groups

Functional pathways with significantly altered abundance between stone history groups. Pathways are summarized based on SEED subsystem 2. Those with LDA effect size greater than 2 are plotted and coloured based on patient group of enrichment.
No history of stones
Previous stone former

- CzcA
- CzcC
- CoZnCd efflux
- CmeA
- CmeB
- CmeC
- RND fusion protein
- Macrolide resistance
- Beta Lactamase (BL)
- Hydrolase (BL superfamily)
- Acriflavine resistance
- Polymyxin resistance
- Aquaporin
- Choline sulfatase
- Pyridoxine metabolism
- Pyridoxine biosynthesis
- L-Ascorbate utilization

Heavy metal resistance
Antibiotic and antiseptic resistance
Regulators of osmotic stress
Decreased oxalate production
Increased oxalate production
Figure 21. Differential proteins between stone history groups

Proteins of interest with significantly differential abundance (LDA effect size >2 [LEfSe] and q score < 0.05 [MaAsLin2]) between stone history groups. Group median is plotted, and all values were normalized to the average frequency in participants with no history of stones. Genes are coloured by functional implication.
Relative abundance of the genus *Oxalobacter* was not different between the healthy control and stone former cohorts, nor between participants with no history of stones and recurrent stone formers (by two tailed Mann-Whitney U test).

**Figure 22. Relative abundance of *Oxalobacter* sp. is not different between participant groups**

Relative abundance of the genus *Oxalobacter* was not different between the healthy control and stone former cohorts, nor between participants with no history of stones and recurrent stone formers (by two tailed Mann-Whitney U test).
3.5 Discussion

Nephrolithiasis is a pervasive pathology, affecting upwards of 10% of the population in North America and increasing in prevalence (Scales Jr., et al., 2012; Tasian et al., 2016). Previous studies investigating the bacterial contribution to this disease have focussed on intestinal bacteria that degrade oxalate, primarily *Oxalobacter formigenes* (Jiang et al., 2011; Liu et al., 2017; Miller et al., 2019; Prokopovich et al., 2007). Several more recent studies have evaluated the gut microbiota in stone disease and describe generalized “dysbiosis” but have been unsuccessful at coming to a consensus with their findings (Miller et al., 2019; Stern et al., 2016; Suryavanshi et al., 2018; Tang et al., 2018; Zampini et al., 2019). Moreover, studies into the urinary and stone microbiota of stone formers has been prefatory, involving few patients or lacking a healthy control comparison group (Barr-Beare et al., 2015; Dornbier et al., 2019; Xie et al., 2020; Zampini et al., 2019). Based on these knowledge gaps left unaddressed by previous investigations, it remained unclear i) exactly how the gut is altered, ii) whether *O. formigenes* colonization is a driving factor, and iii) how the urinary and stone microbiota may contribute to nephrolithiasis. The aim of the current study was to define the urinary and gut microbiome’s involvement in stone disease in the largest multi-omics -based investigation to date.

The results of the current study describe the urinary, stone, and gut microbiota of stone formers and healthy controls. It was confirmed that stone patients had higher urinary oxalate concentrations and an altered urinary microbiota composition compared to healthy controls. The urinary microbiota was enriched in inflammation-associated microbes during surgical stone treatment. A sequence-positive microbiota was found in all stone crystalline compositions and was derived from urogenital microbes, yet compositionally distinct from both pre-operative and intraoperative urine. A lower relative abundance of *O. formigenes* in the gut of stone formers was not found, however, novel functional changes were detected indicative of risk of stone formation which was not dictated by altered dietary consumption. Pathways conferring resistance to toxic compounds were elevated in the gut of stone formers. These findings suggest that the gut is a reservoir of resistance, and likely the root of systemic, aberrant microbial interactions leading to stone formation.
Participant recruitment began in August of 2015 and was completed in January of 2019 after the enrolment of 30 healthy control (HC) participants and 83 stone-forming (SF) patients. The intent was to recruit 200 stone patients however the lower study enrolment rate was a direct reflection of the available patient pool and was still within a sample size suitable to draw conclusions. Despite the Urology Clinic at St. Joseph’s Hospital seeing upwards of 200 stone patients per year, many had taken antibiotics within the last 30 days constituting an exclusion criterion for study participation. In contrast to the lower than expected participant eligibility, the recruitment rate of stone patients approached for study participation was above 90%. Those that declined to participate cited lack of interest.

A full assemblage of samples was collected from 36 out of 83 stone patients; a limiting factor was intraoperative stone collection, since stone fragments were collected only from SF patients who underwent PCNL, rather than ureteroscopic stone removal. All HC participants provided complete sample sets. The dietary questionnaire was introduced as an amendment to the study protocol in 2016 under the guidance of a registered dietician, and therefore the HC and SF participants who had been recruited within the first year prior to its adoption did not complete this. Although this is a limitation, this study was the largest of its kind and involved the most extensive sample and participant characterization.

The urinary oxalate concentrations were significantly higher in SF compared to HC. Urinary levels were calculated to be (mean ± SE) 0.56 ± 0.07 mM, 0.84 ± 0.08 mM, and 0.37 ± 0.04 mM for HC, SF (PreOp), and SF (OR), respectively, after comparing against the concentrations of the known oxalate and creatinine standards. These values are slightly higher than documented urinary levels in healthy and hyperoxaluric patients, indicating a potential error in quantitation due to urinary ascorbate breakdown throughout storage (Baadenhuijsen and Jansen, 1975; Fry and Starkey, 1991; Lemann et al., 1996; Thompson and Fennema, 1971). We therefore did not utilize the exact molarity measurements of urinary oxalate. Instead, the ratio of oxalate [ppm]/creatinine [ppm] was reported, assuming the erroneous methodology was applied to both participant cohorts evenly. With these assumptions in mind, it was concluded that the SF urine was significantly higher in the relative proportion of oxalate compared to HC.
Urinary oxalate is a product of both exogenous dietary consumption of the compound and endogenous production as a terminal product of metabolism (Holmes and Assimos, 1998; Holmes et al., 2001). It is estimated that 20-50% of urinary oxalate results from dietary consumption (Holmes et al., 2001). However, in concordance with previous studies, the present study demonstrated that dietary oxalate consumption was not different between SF and HC (Taylor and Curhan, 2007). Estimates of other relevant nutrients that may play a role in stone formation including water, caffeine, Vitamins B₆, C, D, and K, calcium, sodium, or potassium intake were also not different between cohorts (Han et al., 2015). Although some diet questionnaires tend to exhibit measurement errors due to under-reporting, it was confident that the dietary questionnaire utilized in this study provided accurate assessments of nutrient intake as it has been independently validated (Subar et al., 2001; Subar 2003). Because endogenous oxalate production is largely affected by body size and in this study the cohorts were matched for these characteristics, altered endogenous production was unlikely to be responsible for the elevated oxaluria in SF (Lemann et al., 1996). These findings instead point to altered absorption or bacterial breakdown of dietary oxalate, or its precursors, between cohorts as the divergent factor.

Characterization of the urinary and stone microbiota was completed with 16S rRNA gene sequencing of the V4 region. Whole shotgun metagenomic sequencing is significantly more expensive than 16S rRNA gene sequencing and also requires a much higher DNA yield. Therefore, the urinary samples which were of much lower microbial biomass than the gut samples were assessed with 16S (Hillmann et al., 2018; Whiteside et al., 2015). The urinary microbiota was significantly distinct between HC and SF, and in SF throughout their course of treatment. It has been postulated that both commensal and pathogenic Gardnerella vaginalis strains exist (Harwich et al., 2010). Although this organism is recognized as a pathogen in the vagina, it is often detected in the urine of healthy individuals, and here it was of higher relative abundance in healthy subjects compared to PreOp SF (Gottschick et al., 2017; Mueller et al., 2018). In SF when urine was collected in the OR, the relative abundance of Gardnerella fell along with Lactobacillus and Prevotella spp., while that of Acinetobacter and Escherichia spp. increased significantly compared to the PreOp samples. Explanations for these alterations could include variations in the sampling methodology, the effect of pre-operative and peri-operative antibiotics, or that the urological procedure may have disrupted
and flushed out the *Acinetobacter* and *Escherichia* spp. If the latter, it implies that some genera may be deeply embedded within the uroepithelium (Goneau et al., 2015). *Escherichia* and *Acinetobacter* are known to be associated with infection and inflammation, so their increased detection might lead to additional or longer antibiotic administration (Flores-Mireles et al., 2016; Govender et al., 2019). *Lactobacillus* and *Prevotella* on the other hand are present in typical urotypes of healthy individuals, so their depletion may allow for resistant, deep-seated pathogens to take over the niche (Goneau et al., 2015; Mueller et al., 2018). Such beneficiaries could be pathogenic *G. vaginalis* strains with enhanced adherence and virulence properties (Harwich et al., 2010; Mueller et al., 2018). For such substantial urinary microbiota changes to occur in a short time frame, efforts should be made to understand the reasons and clinical implications.

Although historically only struvite stones were associated with bacteria, recent studies using both culture and molecular techniques have identified their presence in non-struvite kidney stones (Barr-Beare et al., 2015; Dornbier et al., 2019; Tavichakorntrakool et al., 2012; Zampini et al., 2019). Concordantly, after stringent filtering, the present study detected a sequence-positive microbiota in 34 out of 47 stone samples, only 3 of which were composed of struvite. The presence of live bacteria was confirmed with extended quantitative urine culture in a limited number of samples, the results of which were in agreement with the next generation sequencing. Such confirmation is important to overcome criticisms that simply detecting bacterial DNA does not prove the presence of living bacteria (Emerson et al., 2017).

Several genera including *Staphylococcus* and *Streptococcus* have been detected in stones previously (Dornbier et al., 2019; Tavichakorntrakool et al., 2012). This was confirmed in the present study along with numerous novel stone-associated taxa such as *Gardnerella* and *Atopobium* spp. The finding that the stone-associated microbiota was dominated by urinary microbes but was compositionally distinct from both the PreOp and OR SF urine indicates that bacteria inside calculi likely do not derive out of coincidental entrapment in the growing stone matrix, or the resulting community would more closely resemble the PreOp urine. Instead, the results suggest that specific microbes are intimately involved in stone
development, potentially exacerbating crystal nidus formation through inflammation and crystal aggregation (Barr-Beare et al., 2015; Chutipongtanate et al., 2013).

Dogma has held that intestinal colonization by O. formigenes lowers oxaluria and consequently the risk of stone formation, however recent studies have failed to detect differences in the colonization rates between stone-formers and non-formers (Batagello et al., 2018; Kaufman et al., 2008; Kwak et al., 2003; Siener et al., 2013; Ticinesi et al., 2018). In congruence with these studies, we did not detect a difference in the relative abundance of this bacterium in fecal samples from HC and calcium oxalate (CaOx) SF by whole shotgun metagenomic sequencing (Magwira et al., 2012; Miller et al., 2019; Tang et al., 2018; Ticinesi et al., 2018). These results suggest that perhaps the previous emphasis put on O. formigenes colonization has been overstated. It appears that Western society is collectively losing carriage of this bacterium, but this may just be a sign of a progressively ailing population, or the influence of medical practices and prevalent antibiotic use, as opposed to a direct causal factor in stone prevalence (Barnett et al., 2016; PeBenito et al., 2019).

Alpha diversity as well as overall gut microbiota variation were divergent between study participants based on their history of CaOx stone disease and previous stone treatment, rather than between HC and SF cohorts. Indeed, those with a previous history of stones (recurrent stone formers, RSF) had higher relative abundance of various Enterobacteriaceae and the opportunistic pathogens Pseudomonas and Finegoldia, compared to those with no history of stones (NHS) (de Bentzmann and Plésiat, 2011; Goto et al., 2008; Sassone-Corsi et al., 2016). This may be a marker of microbiota damage, precipitated by the repetitive antimicrobial therapy and surgical interventions experienced by the RSF (Ng et al., 2013; Sassone-Corsi et al., 2016; Winter et al., 2013). RSF also had lower relative abundance of the known gut commensal genera Prevotella, Faecalibacterium, and Roseburia compared to NHS (Mangalam et al., 2017; Marchesi et al., 2016; Martín et al., 2014; Tamanai-Shacoori et al., 2017).

Beyond taxonomic differences, many functional changes in the gut of RSF and NHS cohorts were noted. Mapping shotgun metagenomic reads to the SEED database allowed for detection of changes at the level of individual protein encoding genes, as well as from a higher-level view of subsystems, or functionally related protein families (Overbeek et al.,
With this approach, RSF samples were found to be enriched compared to NHS in osmotic stress-related proteins marked by higher relative abundance of aquaporins and choline/betaine transporters. Bacterial aquaporins were first described in 1995, but their characterization to-date remains scant (Calamita et al., 1995, Tong et al., 2019). These membrane channels facilitate the diffusion of water and solutes across cellular membranes, though a role for these proteins in kidney stone disease is undocumented beyond human renal aquaporins and their activation by urinary calcium (Earm et al., 1998). Prokaryotic aquaporins may play a role in bacterial persistence in harsh environments, virulence, and rapid growth in hypoosmotic conditions, and have been characterized in many bacteria including Escherichia, Pseudomonas, and Shigella spp., which were similarly enriched in RSF (Calamita et al., 1998; Kaenjak et al., 1993; Tanghe et al., 2006; Tong et al., 2019). Whether this enrichment in osmotic stress response has a pathogenic role in nephrolithiasis or is a result of altered gut osmolality or toxification warrants further research.

Higher relative abundance of ascorbate utilization and depleted pyridoxine metabolism functional pathways in RSF may be of great consequence, as both of these pathways impact serum and urinary oxalate levels. These pathways may have been previously overlooked by groups solely focussed on direct oxalate breakdown. Ascorbate, or vitamin C, is a precursor to oxalate and its intake is associated with risk of incident kidney stones (Ferraro et al., 2016). Elevated utilization of vitamin C by bacteria in the RSF gut may lead to higher intestinal levels of absorbable oxalate, despite no difference in vitamin C or oxalate dietary consumption between cohorts in this study (Baxmann et al., 2003; Eddy and Ingram, 1953; Stack et al., 2020). This ascorbate utilization has been documented in Pseudomonas and Acinetobacter spp. among others, both of which were elevated in (R)SF patients (Stack et al., 2020). Thus, for some people suffering from recurrent stone formation, the levels of gut microbiota mediated ascorbate utilization (by qPCR or ELISA) might be worth determining.

Pyridoxine, also known as vitamin B₆, is a cofactor necessary for the enzyme alanine-glyoxylate aminotransferase’s conversion of glyoxalate to glycine. Deficiency of this vitamin has been associated with elevated oxaluria (Curhan et al., 1999; Ferraro et al., 2017). Bacteria can biosynthesize B₆, and also convert it to the active form, pyridoxal 5’ phosphate (PLP) (Dempsey, 1967; Eliot and Kirsch, 2004; Magnúsdóttir et al., 2015; Strohmeier et al.,
Gut colonizing species within the genera *Prevotella*, *Corynebacterium*, and *Clostridium* are capable of B$_6$ and PLP biosynthesis, and these genera were of relatively higher abundance in the gut of NHS compared to RSF (Jochmann et al., 2011; Magnúsdóttir et al., 2015; Yoshii et al., 2019). The finding that antibiotic use can lead to B$_6$ deficiency again raises the issue of the extent to which antibiotics influence recurrence of stone formation (Levy, 1969; Meletis and Zabriskie, 2007; Snider, 1980). Although vitamin B$_6$ intake was not different between participant cohorts, RSF may have reduced microbiologically synthesized pyridoxine, or lower levels of the microbiologically activated PLP, ultimately leading to increased urinary oxalate. Despite B$_6$ supplementation being previously tested as a therapy for nephrolithiasis with some success, its use has not been widely implemented, nor has the microbial contribution to this vitamin been evaluated in the context of stone formers (Mitwalli et al., 1988; Pearle et al., 2014; Prien and Gershoff, 1974; Rao and Choudhary, 2005). Serum and fecal PLP concentration should be collected in future studies of the gut microbiome and nephrolithiasis to identify the keystone microbes necessary for host pyridoxine acquisition and confirm whether these are depleted in RSF.

The gut microbiota of RSF was enriched in functional pathways associated with resistance to toxic compounds including heavy metals and a variety of antibiotic and antiseptic classes. Specifically, the cobalt/zinc/cadmium efflux system encoded on the *czcCBA* operon was significantly enriched in RSF (Liu et al., 2015). Interestingly, both zinc and cadmium exposure have been associated with elevated stone risk (Guo et al., 2018; Tang et al., 2012). Although biological levels of these metals were not investigated in the present study, because serum levels of these metals are largely derived from dietary consumption, they were likely not different between the cohorts due to their comparable diets (Canada Environmental Protection Act, 2007; Dabeka and McKenzie, 1992). However, if the gut microbiota of RSF effluxes more of these metals into the intestinal lumen instead of providing a “quenching” effect as some bacteria do, increased amounts may be bioavailable for unfavourable absorption by the host (Bisanz et al., 2014; Daisley et al., 2018). For this reason, heavy metals should be evaluated in future studies of the gut microbiome and stone formation; if cadmium-resistant bacteria in the gut such as *Pseudomonas* spp. are contributing to a biologically relevant increase of serum levels in stone formers, this cadmium may be
aggravating crystal precipitation and stone formation in the kidney (Liu et al., 2015; Thomas et al., 2013).

The cmeABC operon encodes a multidrug efflux pump of the RND superfamily and confers resistance to a broad range of antibiotics including ciprofloxacin (Fernando and Kumar, 2013; Lin et al., 2002; Yan et al., 2006). This operon as well as genes encoding resistance to β-lactams, macrolides, polymyxins, and the topical antiseptic acriflavine were significantly elevated in RSF compared to NHS. It is known that the use of antibiotics, particularly in early life, can have lasting impacts on the microbiome, host metabolism, and immunity (Cho et al., 2012; Cox et al., 2014; Ruiz et al., 2017). The recent report of a direct link between oral antibiotic use and the risk of nephrolithiasis highlighted sulfas, cephalosporins, fluoroquinolones, nitrofurantoin, and β-lactams as problematic, especially with recent and younger age exposures (Tasian et al., 2018).

Although neither cohort had used antibiotics within 30 days prior to providing their fecal sample in the present study, antibiotics were prescribed significantly more recently in SF than HC. Indeed, throughout the course of surgical stone treatment, patients were treated with perioperative antibiotics, and those with planned PCNL additionally took pre-surgical prophylactic antibiotics. The data suggest this practice may be enriching for Gammaproteobacteria including Enterobacteriaceae and antibiotic-resistance in the gut of RSF subjects, as they were administered antibiotics at each previous surgical stone treatment. As the gut is a bacterial reservoir for the urinary tract, antibiotic-resistant strains may have ascended from the rectum (Magruder et al., 2019; Yamamoto et al., 1997). In support of this, Acinetobacter spp. (of class Gammaproteobacteria), often multidrug resistant and considered serious uropathogens with increasing nosocomial disease burden, were enriched in SF OR urine (Bergogne-Bérézin et al., 1996; Di Venanzio et al., 2019; Jiménez-Guerra et al., 2018). Therefore, patients with previous surgical intervention for calculi have likely experienced antibiotic-induced, functional changes to their gut microbiota resulting in the enrichment of potentially pathogenic, multidrug resistant microbes.

We therefore propose, based on these collective findings and in corroboration with the work of others, that antibiotics induce a widespread assault on the gut and urinary microbiota, driving the depletion of beneficial gut microbes such as Faecalibacterium and Lactobacillus
spp., while simultaneously enriching for antibiotic resistant uropathogens. This ultimately leads to disrupted oxalate homeostasis due to altered microbiota-mediated micronutrient biosynthesis and utilization, intestinal toxin accumulation, and the blooming of uropathogens in the bladder via the interconnected pelvic floor microbiota (Moreno et al., 2006; Thomas-White et al., 2018; Yamamoto et al., 1997). These biological consequences all collectively impact stone formation, and the repeated microbiome assaults inflicted by surgical stone treatment and its accompanying antibiotic use perpetuate the cyclic recurrence of the disease.

With this in mind, future management of nephrolithiasis should comprise both prevention of microbiome disturbance and the use of agents for subsequent restoration of homeostasis. Antibiotic stewardship is a first and obligatory step in preventing the unnecessary disruption of microbial balance (Mossanen et al., 2014; Siemens and Nickel, 2015). Although often required to avert systemic infection, there is still debate over antibiotic prophylaxis for PCNL in low-risk patients (Chew et al., 2018). In such cases, avoidance of antibiotics may reduce the progression of microbial dysbiosis and its subsequent ramifications for stone formation. In addition, microbial restoration therapies such as probiotics and prebiotics could replenish organisms associated with a healthy urinary tract and may offer relief to recurrent stone formers that have already experienced substantial microbiota damage (Bustamante et al., 2020; Collins et al., 2018; Falagas et al., 2006; Stapleton et al., 2011). Such restoration may even include fecal microbiota transplant (FMT), based on early evidence of efficacy against recurrent urinary tract infection (Hocquart et al., 2019; Tariq et al., 2017). Where previous studies implementing supplementation of a single microbe (as has been attempted with O. formigenes) have yielded mixed results, FMT may hold promise (Duncan et al., 2002; Ellis et al., 2015; Jairath et al., 2015; Stern et al., 2019). Future studies should investigate the therapeutic potential of FMT for nephrolithiasis, where it may restore the robust community and interactive network of beneficial microbes that becomes depleted in recurrent stone formers (Miller et al., 2017; Stern et al., 2019). It would also be important to validate how long the effects of an FMT persist in stone formers, and the extent to which altering the gut microbiome spills over to the urinary tract.

In conclusion, the results of the current study demonstrate how antimicrobial use inherent in surgical stone management depletes beneficial microbes in the gut and urinary tract, causing
a decreased capacity to maintain oxalate homeostasis and the development of an intestinal reservoir of antibiotic resistant uropathogens. That these microbes are detected within stone fragments further suggests that elevated urinary oxalate in concert with the presence of inflammation-mediating microbes in the upper urinary tract leads to the development of crystal nidi and aggravates recurrent stone development. We postulate that the previous emphasis of direct oxalate degradation and *O. formigenes* colonization status in the gut has been overstated. Rather, a novel, inconspicuous method of oxalate homeostasis is causative and dependent on microbial micronutrient metabolism and biosynthesis. If the diversity and robust functional potential of the healthy human microbiome is repeatedly assaulted by the average Westernized lifestyle via diet, antibiotic use, and other environmental factors, then kidney stone prevalence will continue to increase (Lozupone *et al*., 2012; Tasian *et al*., 2016). Reversing this trend through microbiome-targeted therapeutic applications should be studied as a means to prevent this debilitating condition.

### 3.6 References


Chapter 4

4 Characterizing the microbial communities associated with ureteral stents

4.1 Abstract

Ureteral stents are commonly used in urological practice and can often become bacterially colonized and encrusted, leading to clinical complications. Despite recent discovery and characterization of the healthy urinary microbiota, stent-associated bacteria and their impact on encrustation are largely underexplored. We profiled the bacterial communities of ureteral stents and mid-stream urine from a single clinical centre over a one-year period. 16S rRNA gene sequencing was utilized to determine factors that impacted variation in the microbial communities. Two hundred and forty-one patients were examined, including typical short-term stent insertion cases, as well as atypical cases of bilateral stenting, long-term indwelling stents, patients with multiple sequential stents over time, and antegrade stent placement. Indwelling time, age, and various patient comorbidities including diabetes and IBS/IBD impacted the stent microbiota, whereas antibiotic treatment, UTI, and stent placement method did not. The stent microbiota originates from adhesion of urinary microbes and subsequently diverges to a distinct and reproducible population, thereby negating the urine as an accurate biomarker for stent encrustation or microbiota. Urological practice should reconsider standalone prophylactic antibiotic use in favour of tailored therapies based on patient comorbidities in efforts to minimize bacterial burden, encrustation, and complications of ureteral stents.
4.2 Introduction

Ureteral stents are hollow conduits placed in the ureter from the renal pelvis to the bladder and are commonly used in urological practice to maintain urine drainage, which can be impeded by obstruction caused by urolithiasis, stricture, or malignancy. Termed double pigtail or double J, typical stents involve a curl at either end to prevent displacement. They are typically composed of polyurethane, silicon, and new combination polymers (Chew and Denstedt, 2004; Mosayyebi et al., 2018). Due to constant contact with the urine, deposition of urinary crystals and formation of bacterial biofilms on stents are common (Reid et al., 1992; Zumstein et al., 2017). The formation of these encrustations can lead to complications including infection, failure of the stent to drain urine, more frequent device exchanges, and subsequent difficulty with removal. Indwelling ureteral stents have been associated with the development of urinary tract infections (UTIs), and in more severe cases, pyelonephritis or urosepsis which may be related to single species or polymicrobial biofilms attached to the stent (Lange et al., 2015).

The urinary tract harbours a unique microbiota which is distinct from that of the gut in composition and is of much lower abundance (Whiteside et al., 2015; Wolfe et al., 2012). Based on recent evidence, it is likely that the different sites and tissues throughout this system have different microbiotas (Barr-Beare et al., 2015; Cavarretta et al., 2017; Wolfe et al., 2012). The biofilms that form on stents may originate from this microbiota or contamination during insertion of the device. Regardless of their origin, the development of biofilms on these devices illustrates that even very low numbers of bacteria can quickly take advantage of the niche-altering foreign material to expand their populations. Previous studies in stent patients have identified bacterial colonization rates from 70 – 90% (Rahmann et al., 2010; Reid et al., 1992). Bacteriuria can be common in upwards of 20% of patients with stents, and Escherichia coli is often the most commonly cultured and identified organism (Rahmann, et al., 2010). Bacterial isolates derived from stent biofilms of clinical origin often demonstrate resistance to multiple antibiotics, and antibiotic prophylaxis or concomitant antibiotic administration does not appear to reduce the incidence of stent-related symptoms or urinary tract infection incidence or severity (Chatterjee et al., 2014; Chew and Lange, 2009; Moltzahn et al., 2013; Paz et al., 2005). Due to these findings, the use of antibiotic
prophylaxis for stents is controversial, and it is unclear how these compounds may impact the urinary microbiota during stenting.

The purpose of this study was to elucidate how the urinary microbiota and other host factors impact bacterial colonization and encrustation of indwelling ureteral stents. As such, we utilized 16S rRNA gene sequencing and scanning electron microscopy to characterize the urine and stent microbiota from 241 patients that were sampled from a single urology centre. The large sample size and complementary nature of the samples provide the first high resolution insight into bacterial attachment to ureteral stents under different clinical scenarios. This work may help clinicians and scientists to have a better understanding of what types of bacteria colonize or adhere to ureteral stents, factors that increase or decrease the likelihood of stent colonization and encrustation, and provision of best management practises.

4.3  Materials and Methods

4.3.1  Study design and clinical sample collection

Ureteral double-J stent patients were recruited from the Urology Department at St. Joseph’s Hospital in London, Ontario. Ethical approval for the study was granted by Lawson Health Research Institute and the Health Sciences Research Ethics Board at the University of Western Ontario (REB #107941, Appendix C) in London, Ontario. Written consent was obtained from all the study participants at the time of study inclusion and the methods were carried out in accordance with the approved guidelines.

The study proposed to recruit up to 500 participants over a 2-year period; at study conclusion 241 participants had been enrolled. Inclusion and exclusion criteria for the participants are provided in Table 12. All patients that met the inclusion criteria were recruited to the study during regularly scheduled clinic appointments. Upon recruitment, patients were asked about relevant demographic and medical history including antibiotic usage and their history of urinary tract infections. Following enrolment, participants provided a mid-stream urine sample. Stents were collected during cystoscopy (either in-clinic or OR) and placed by the surgeon into a sterile urine collection cup (Figure 23).
4.3.2 Clinical sample processing and DNA extraction

Urine samples were processed in two portions within 6-hours of their collection. Where possible, 10 mL of whole urine was collected and frozen at -20 °C for future high-performance liquid chromatography (HPLC) analyses of urinary metabolites. The remainder was stored for future 16S rRNA gene sequencing: the entire remaining volume of urine was centrifuged for 10 minutes at 5,000 x g, after which the supernatant was decanted off and the pellet was stored dry at -20 °C until DNA extraction. If the total urine volume was under 25 mL, only 2 mL of whole urine was reserved for calcium oxalate quantitation by HPLC. The urine volume that resulted in the pellet for 16S rRNA gene sequencing was recorded to identify confounding factors in the downstream sequencing analysis associated with processing conditions.

Within 6 hours of their collection, stents were frozen at -20 °C and stored until DNA extraction. On the day of DNA extraction, the stents were thawed and processed in a sterile biosafety hood. A scalpel sterilized with RNase AWAY™ (Thermo Scientific, Waltham, MA, USA) was utilized to slice two x 1 cm portions from both the proximal and distal curls of the stents (Figure 23). One 1 cm slice from each curl was utilized for 16S rRNA gene sequencing: using tweezers sterilized with RNase AWAY™, 1 mL of nuclease free water (Ambion, Mississauga, ON, CAN) was gently rinsed over the external surface of the stent and into the inner lumen of the stent where possible (this was sometimes inhibited by encrustation). The rinsed cut was then directly transferred into the bead plate of the DNeasy PowerSoil HTP 96 Kit utilized for DNA extraction (Qiagen, Toronto, ON, CAN). A second 1 cm cut segment was reserved for scanning electron microscopy (SEM): both the internal lumen and exterior of the stent were of interest for imaging so the stent cut was sliced lengthwise and both halves were transferred to a sterile 1.5 mL Eppendorf tube for SEM preparation.
Table 12. Inclusion and exclusion criteria for study participation

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
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<tr>
<td>At least 18 years of age</td>
<td>In the opinion of the treating urologist, it is not in the patient’s best interest to participate</td>
</tr>
<tr>
<td>Has a ureteric stent scheduled for removal</td>
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<tr>
<td>Able and willing to provide informed consent</td>
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Figure 23. Schematic of stent sample processing

Stents were collected by the surgeon and placed into a sterile urine collection cup. A sterile scalpel was utilized to slice two x 1 cm portions from both the proximal and distal curls of the stents. One 1 cm slice from each curl was utilized for SEM imaging, the other for 16S rRNA gene sequencing. The portion for 16S sequencing was gently rinsed externally and internally where possible with nuclease-free water over a sterile reservoir and added directly to the DNA extraction plate. Image templates from Servier Medical Art by Servier were used and modified under the Creative Commons Attribution 3.0 Unported License.
For DNA extraction, frozen urine pellets were thawed and suspended in 100 μL of nuclease-free water (Ambion), then pipetted into individual wells of the PowerSoil HTP bead plate with PCR-grade filter tips (FroggaBio, Toronto, ON, CAN). Two wells in every plate were left empty and acted as negative controls. Two positive controls, or spikes, were added to each plate and were 100 μL of pure bacterial culture: Spike 1 was *Escherichia coli* strain DH5α, and Spike 2 was *Staphylococcus aureus* strain Newman. For preparation of the spikes, a single colony of the bacteria was inoculated into 10 mL of Luria-Bertani (LB) broth and grown overnight at 37 °C. One hundred 100 μL aliquots of the overnight cultures were portioned into 1.5 mL Eppendorf tubes and frozen at -80 °C. For each DNA extraction plate, a single tube of both spikes was thawed and pipetted directly into the PowerSoil HTP bead plate with PCR-grade filter tips.

DNA was isolated from urine and stent samples using the DNeasy PowerSoil HTP 96 Kit according to the manufacturer’s instructions. Briefly, 750 μL of bead solution and 60 μL of Solution C1 were added to the bead plate loaded with all samples and controls. Plates were shaken for 20 minutes at speed 20 using the MoBio 96-well plate shaker (Qiagen), then centrifuged for 10 minutes (all centrifuge steps were conducted at room temperature at 2250 x g). Five hundred μL of the supernatant was added to a fresh plate with 250 μL Solution C2 and mixed by pipetting. The plates were incubated at 4 °C for 10 minutes followed by centrifugation for 10-minutes. The resulting supernatant was then transferred to a fresh plate and the centrifugation step was repeated. Approximately 600 μL of the solution was then transferred to a fresh plate containing 200 μL Solution C3 and mixed by pipetting. The plate was incubated at 4 °C for 10 minutes followed by centrifugation for 10-minutes. The entire volume, with the exception of the pellet, was again transferred to a fresh plate for centrifugation. Carefully avoiding the loose residual pellet, 650 μL was transferred from each well to a fresh plate containing 1300 μL Solution C4. The plates were then sealed with sealing tape and stored at 4°C overnight. The following day the plates were briefly centrifuged and sealing tape was removed. Solution in the wells was then mixed by pipetting and 500 μL was transferred to a Spin Plate, which was centrifuged for 5 minutes. The flow-through was discarded, and this step was repeated until the entire sample-Solution C4 mix was processed through the Spin Plate. A volume of 500 μL Solution C5-D was then added to the Spin Plate and centrifuged twice for ten minutes, where the flow-through was discarded.
between spins. 100 μL Solution C6 was then added to the Spin Plate, incubated at room temperature in the biosafety hood for 10 minutes and the resulting DNA was eluted to a Microplate via centrifugation for 15 minutes. DNA was stored at -20 °C until PCR amplification.

4.3.3 16S rRNA gene amplification and sequencing

PCR amplification was completed using the Earth Microbiome universal primers, 515F and 806R, which are specific for the V4 variable region of the 16S rRNA gene (Figure 4). Primers contained an Illumina adapter, followed by four random nucleotides, one of 24 unique 12-mer barcodes, and the annealing left or right primer (Figure 4) (Parada et al., 2016). Primers and barcode sequences are listed in Table 7. PCR reagent set-up was performed using a Biomek® 3000 Laboratory Automation Workstation (Beckman-Coulter, Mississauga, ON, CAN). Ten μL of each left and right-barcoded primers (3.2 pMole/μL) were arrayed in 96-well plates (Axygen-Corning, Oneota, NY, USA) such that each well contained a unique combination of left- and right- barcodes (up to a maximum of 576 unique combinations). Two μL of DNA template was added to the primer plate, followed by 20 μL of Promega GoTaq hot-start colourless master mix (Promega, Madison WI, USA). The reaction was briefly mixed by pipetting, then plates were sealed with foil plate covers (Axygen-Corning) and centrifuged for 2 minutes at room temperature at 2250 x g.

Amplification was carried out using an Eppendorf thermal cycler (Eppendorf, Mississauga, ON, CAN), where the lid temperature was maintained at 104 °C. An initial warm-up of 95 °C for 4 minutes was utilized to activate the GoTaq, followed by 25 cycles of 1 minute each of 95 °C, 52 °C, and 72 °C.

DNA extraction was completed across a total of 11 x 96-well plates, as in some cases stent samples were extracted in parallel with samples from other experiments. Due to the total number of samples exceeding the number of unique barcode combinations, two Illumina MiSeq runs were completed to accommodate the sequencing of all the samples (Illumina Inc., San Diego, CA, USA). In order to identify potential batch effects between the two sequencing runs, several samples and controls were sequenced on both runs. In total, accounting for doubly sequenced samples, 822 samples were sequenced across 9 PCR plates.
(5 x 96-well plates containing 438 samples on the first MiSeq run, 4 plates containing 384 samples on the second). Sequencing was carried out at the London Regional Genomics Centre (http://www.lrgc.ca; London, ON, CAN). Amplicons were quantified using pico green (Quant-It; Life Technologies, Burlington, ON) and pooled at equimolar concentrations before cleanup (QIAquick PCR clean up; Qiagen, Germantown, MD). Using the 600-cycle MiSeq Reagent Kit (Illumina Inc.), paired-end sequencing was carried out as \( 2 \times 260 \) cycles with the addition of 5% \( \Phi X-174 \) at a cluster density of \(~1100\). Data was exported as raw fastq files (uploaded to NCBI Sequence Read Archive, BioProject ID #PRJNA601180).

From the two sequencing runs, run 1 contained 438 samples and yielded a total of 16,211,576 reads, ranging from 419 to 358,493 reads per sample. Run 2, containing 384 samples, yielded a total of 10,424,180 reads, ranging from 168 to 400,010 reads per sample. An average of 20.8% and 18.8% of reads were removed from each sample in Runs 1 and 2, respectively, following quality filtering performed utilizing the DADA2 pipeline (Callahan et al., 2016). The remaining filtered reads from the two runs (14,477,624 and 9,697,990) were then merged by amplicon sequence variants (SVs). SVs that were only detected in one of the two runs were removed. Samples and SVs were then further pruned such that the final dataset utilized in all downstream analyses retained samples with greater than 1,000 filtered reads, SVs present at 1% relative abundance in any sample, and SVs with greater than 10,000 total reads across all samples in both runs. This cleaning reduced the dimensions of the dataset from 460 SVs and 822 samples down to 43 SVs and 711 samples. The remaining 43 SVs were assigned taxonomy with the SILVA (v132) training set, and a further 5 SVs were removed due to their alignment to human mitochondrial sequences (Quast et al., 2013).

Downstream analysis was performed with ALDEex2, MaAslin2, Vegan, and core R packages (Fernandes et al., 2013; Morgan et al., 2012; Oksanen et al., 2019; R Core team, 2019).

### 4.3.4 Scanning electron microscopy and X-ray diffraction spectroscopy

One-centimetre stent sections were cut open lengthwise with a sterile razor and mounted upon aluminum stubs such that one half exposed the inner lumen, and the other half exposed the external surface. They were then gently rinsed with DI water to remove salt precipitation
prior to SEM and X-ray diffraction spectroscopy analysis at the Western University Nanofabrication Facility (https://nanofab.uwo.ca).

4.4 Results

4.4.1 Study recruitment and participant demographics

Participant recruitment began in July of 2016 and concluded in May of 2017 after the recruitment of 241 stent patients. Their average age was 59 years; 122 were female and 119 were male. Patient demographic characteristics are summarized in Table 13. The majority of samples were collected from typical stenting events, where one double-J stent links between one of the kidneys and the bladder. However, cases of bilateral (stents between both the left and right kidney to the bladder), longitudinal (receiving multiple consecutive devices), antegrade (placed downwards from the kidney percutaneously rather than upwards from the urethra), uncommonly long indwelling times, and various encrustation levels were also examined. The majority of study participants had an indwelling stent placed for treatment related to stone disease, though in 22 participants stents were necessitated for other reasons including radiation-induced ureteral stricture and the presence of retroperitoneal masses. Stent indwelling time ranged from 2 to 394 days.
### Table 13. Demographic and clinical characteristics of study participants

<table>
<thead>
<tr>
<th>Participant characteristic</th>
<th>N = 241 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>59.01 ± 13.84 (range 22 – 90)</td>
</tr>
<tr>
<td>Sex</td>
<td>122 females (50.6), 119 males (49.4)</td>
</tr>
<tr>
<td>Indwelling time</td>
<td>22.79 ± 34.61 days (range 2 – 394)</td>
</tr>
<tr>
<td>BMI</td>
<td>31.04 ± 7.64 (range 17.00 – 60.00)</td>
</tr>
<tr>
<td>Reason for stent placement:</td>
<td>Urolithiasis 219 (90.9)</td>
</tr>
<tr>
<td></td>
<td>Stricture 5 (2.1)</td>
</tr>
<tr>
<td></td>
<td>Mass 10 (4.1)</td>
</tr>
<tr>
<td></td>
<td>Other 7 (2.9)</td>
</tr>
<tr>
<td>Stent placement method:</td>
<td>Retrograde 219 (90.9)</td>
</tr>
<tr>
<td></td>
<td>Antegrade 22 (9.1)</td>
</tr>
<tr>
<td>Patients with bilateral stents</td>
<td>11</td>
</tr>
<tr>
<td>Patients with multiple sequential stents over time</td>
<td>11 (9 patients with 2 devices, 2 patients with 3 devices)</td>
</tr>
<tr>
<td>Time between sequential stent placements</td>
<td>63.5 ± 28.6 days (range 25 – 105)</td>
</tr>
<tr>
<td>Use of antibiotics within the last 30 days from stent collection</td>
<td>225 (93.4)</td>
</tr>
<tr>
<td>Previous history of UTI</td>
<td>99 (41.1)</td>
</tr>
<tr>
<td>UTI within 7 days of stent placement or whilst indwelling</td>
<td>37 (15.4)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>54 (22.4)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>99 (41.1)</td>
</tr>
<tr>
<td>Hyperuricemia</td>
<td>16 (6.6)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>122 (50.6)</td>
</tr>
<tr>
<td>IBS</td>
<td>17 (7.1)</td>
</tr>
<tr>
<td>IBD</td>
<td>28 (11.6)</td>
</tr>
<tr>
<td></td>
<td>Crohn’s disease 8 (3.3)</td>
</tr>
<tr>
<td></td>
<td>Ulcerative colitis 20 (8.3)</td>
</tr>
<tr>
<td>Pulmonary disease</td>
<td>72 (29.9)</td>
</tr>
</tbody>
</table>
4.4.2 16S rRNA gene sequencing

Stringent bioinformatic filtering was performed such that 711 samples and 43 amplicon sequence variants (SVs) were maintained for downstream analysis. The most abundant SVs in stent and urine samples corresponded to the bacterial genera *Staphylococcus*, *Enterococcus*, *Lactobacillus*, and *Escherichia* (Table 14). The clinical samples (not including positive and negative controls) contained an average of 13.5 SVs, ranging from 3 to 31. There was a positive correlation between read count and observed SVs (Figure 24A); the correlation coefficient was 0.44 (P < 0.0001, 95% CI 0.38 - 0.50). Urine samples had significantly more SVs observed (Figure 24B) and higher total read count (Figure 24C) compared to stent samples (P = 0.0063 and P < 0.0001, respectively).

The sequence counts were centred log ratio (CLR) transformed, generating sample-wise Aitchison distances (Gloor *et al.*, 2017). A heatmap representing the relative abundance of CLR-transformed samples was generated based on the Aitchison distance average linkage clustering (Figure 25). The differences in microbiota composition at the genus level were not driven by sex or sample type (urine or stent). This was confirmed with a Benjamini-Hochberg corrected Welch’s t-test and principal component analysis (PCA) performed on the log-ratio transformed data at SV-level (Figure 26), where all samples and sex subsets did not separate by sample type (Figure 27A). These findings demonstrate that the same microbes dominate both stent and urine samples from a single patient, and therefore the stent microbiota is likely to be urinary derived.

Although sample types were dominated by similar organisms, stent samples were further compared based on curl position to determine if the two curls (proximal curl in the kidney, and distal in the bladder) had a distinct microbial profile compared to that of the patient’s urine (Figure 27B). Specifically, beta diversity was measured by Aitchison distance to evaluate the distance between proximal and distal curls from each stent, as well as between the stent curls and the urine. Stent curls had significantly shorter distances between proximal and distal curls versus the further distance between stent curls to the urine sample. Thus, microbiota composition of the stent curls was more similar to each other than either curl to the urine, indicating the presence of a stent-specific microbiota that doesn’t directly reflect the composition of the urine.
From the devices recovered from participants with multiple sequentially placed stents, many of the same organisms were detected within the same individual over time (Figures 28 and 29). Upon PCA, samples from the same individual generally clustered together (Figure 29A). Distance between samples from the same participants at different time points was shorter than between samples from different participants (Figure 29B, Kruskall-Wallis test with Dunn’s multiple comparisons, \( P = 0.022 \)). There were no significant effects of visit number on the samples (Benjamini-Hochberg corrected Wilcoxon rank sum test, data not shown). Thus, on a per-patient basis, the stent microbiota is a reproducible community over time, even over the course of up to 150 days.

The microbiota of bilateral stents did not differ significantly, as determined from eleven subjects (Figures 30 and 31). Within patients, both proximal and distal ends of bilateral stents clustered separately from the urine (Figure 31A). Intraindividual samples were closer together than interindividual samples (Figure 31B). There was greatest spread between stent and urine samples from the same individual, and the distance between stent samples was the shortest (Figure 31B), again indicating the presence of a distinct and reproducible stent-specific microbiota.

To determine if patient and sample attributes (metadata) drove microbiota variation, CLR-transformed sample-wise Aitchison distances were evaluated (Oksanen et al., 2019). With this approach, several metadata factors were determined to be microbiota confounders, including stent indwelling time and patient comorbidities (Table 15). These confounders were subsequently adjusted for, and several statistically significant drivers of microbiota variation remained between metadata characteristics and taxonomic features as determined using a general linear model, including patient age, BMI, stent indwelling time, pulmonary disease, hypertension, diabetes, IBS, IBD, and hyperlipidemia (Table 16) (Morgan et al., 2012).

To determine how the degree of encrustation impacted microbial composition, stents were categorized based on visible encrustation level (Table 17). There was a correlation between the degree of stent encrustation and the amount of time stents were indwelling (Figure 32A); the correlation coefficient was 0.32 (\( P < 0.0001 \), 95% CI 0.20 – 0.37). Shannon’s index of alpha diversity was negatively correlated with the degree of stent encrustation (Figure 32B);
the correlation coefficient was -0.14 ($P = 0.0005, 95\% CI -0.22 \text{ to} -0.03$). Shannon’s index was also significantly lower in grade-3 encrusted stents compared to grade-0 (Figure 32C; Kruskall-Wallis with Dunn’s multiple comparisons, $P = 0.027$). This suggests that the longer a stent is indwelling, the more likely it will be to become encrusted by a less diverse microbial community.

Ten study participants had stents indwelling for greater than two months; these participants were determined to be outliers, having stents significantly longer than the average indwelling time of 23 days (“ROUT” method of outlier detection, $Q = 0.1\%$) (Motulsky and Brown, 2006). Microbial communities of participants with “long-term” stents indwelling for 60 or more days were evaluated (Figure 33). The microbiota of these patients was not significantly different when compared to all samples, or to samples from the ten participants with the shortest indwelling durations (Benjamini-Hochberg corrected Wilcoxon rank sum test, data not shown). However, as indwelling time increased, relative abundance of the genera *Finegoldia* and *Porphyromonas* increased, and *Enterococcus* and *Escherichia* decreased (Table 16).

Antibiotic usage was widespread amongst participants; about 93\% had used antibiotics within 30 days of sample collection (Table 13). However, the microbiota of the few participants without recent antibiotic usage was not significantly different than the majority (Table 16, Figure 34A and by Benjamini-Hochberg corrected Wilcoxon rank sum test, data not shown). These participants also did not differ by encrustation grade or alpha diversity (Figure 34B-C; Mann-Whitney U test, $P = 0.091$ and $P = 0.25$ respectively).

Stents were evaluated based on their placement method. The majority (90.9\%, Table 13) of stents were placed in a retrograde manner, however the microbiota of the stents placed antegrade (i.e. during percutaneous nephrolithotomy or nephroscopy) was not significantly different than those placed retrograde (Table 16, Figure 34D). These participants also did not differ by encrustation grade or alpha diversity (Figure 34E-F; Mann-Whitney U test, $P = 0.90$ and $P = 0.95$ respectively).

About 15\% of patients had culture positive UTIs within 7 days of stent placement, or throughout the stent indwelling period (Table 13). The microbiota and degree of stent
encrustation in these patients was not significantly different than those without UTIs (Figure 34G and H), however alpha diversity was lower for patients with UTIs (Figure 34I, Mann-Whitney U test, $P = 0.002$).
Table 14. Ten most abundant sequence variants in urine and stent samples

**Urine samples**

<table>
<thead>
<tr>
<th>Rank</th>
<th>Sequence Variant</th>
<th>Corresponding Genus</th>
<th>Average abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SV_603</td>
<td><em>Staphylococcus</em></td>
<td>18.09</td>
</tr>
<tr>
<td>2</td>
<td>SV_705</td>
<td><em>Enterococcus</em></td>
<td>17.53</td>
</tr>
<tr>
<td>3</td>
<td>SV_709</td>
<td><em>Lactobacillus</em></td>
<td>11.75</td>
</tr>
<tr>
<td>4</td>
<td>SV_213</td>
<td><em>Escherichia</em></td>
<td>11.61</td>
</tr>
<tr>
<td>5</td>
<td>SV_695</td>
<td><em>Lactobacillus</em></td>
<td>6.13</td>
</tr>
<tr>
<td>6</td>
<td>SV_713</td>
<td><em>Lactobacillus</em></td>
<td>5.20</td>
</tr>
<tr>
<td>7</td>
<td>SV_108</td>
<td><em>Prevotella</em></td>
<td>2.42</td>
</tr>
<tr>
<td>8</td>
<td>SV_60</td>
<td><em>Pseudomonas</em></td>
<td>2.28</td>
</tr>
<tr>
<td>9</td>
<td>SV_40</td>
<td><em>Ureaplasma</em></td>
<td>2.10</td>
</tr>
<tr>
<td>10</td>
<td>SV_208</td>
<td><em>Citrobacter</em></td>
<td>1.63</td>
</tr>
</tbody>
</table>

**Stent samples**

<table>
<thead>
<tr>
<th>Rank</th>
<th>Sequence Variant</th>
<th>Corresponding Genus</th>
<th>Average abundance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SV_705</td>
<td><em>Enterococcus</em></td>
<td>21.43</td>
</tr>
<tr>
<td>2</td>
<td>SV_603</td>
<td><em>Staphylococcus</em></td>
<td>18.73</td>
</tr>
<tr>
<td>3</td>
<td>SV_213</td>
<td><em>Escherichia</em></td>
<td>16.21</td>
</tr>
<tr>
<td>4</td>
<td>SV_709</td>
<td><em>Lactobacillus</em></td>
<td>9.15</td>
</tr>
<tr>
<td>5</td>
<td>SV_713</td>
<td><em>Lactobacillus</em></td>
<td>4.98</td>
</tr>
<tr>
<td>6</td>
<td>SV_695</td>
<td><em>Lactobacillus</em></td>
<td>4.24</td>
</tr>
<tr>
<td>7</td>
<td>SV_60</td>
<td><em>Pseudomonas</em></td>
<td>3.67</td>
</tr>
<tr>
<td>8</td>
<td>SV_208</td>
<td><em>Citrobacter</em></td>
<td>2.34</td>
</tr>
<tr>
<td>9</td>
<td>SV_40</td>
<td><em>Ureaplasma</em></td>
<td>1.90</td>
</tr>
<tr>
<td>10</td>
<td>SV_648</td>
<td><em>Veillonella</em></td>
<td>1.66</td>
</tr>
</tbody>
</table>
A) Sample read count was positively correlated with the number of observed SVs, as calculated by the Spearman correlation coefficient ($r = 0.44$, $P < 0.0001$). $R^2$ was calculated as the least-squares fit of the semilog line. B) The number of SVs observed was higher in urine samples compared to stents (Mann-Whitney U test, $P = 0.0063$). C) The total read count was higher in urine samples compared to stents (Mann-Whitney U test, $P < 0.0001$). Box plot whiskers represent minimum and maximum.

Figure 24. Assessment of sample read count and microbiota richness as measured by the number of observed sequence variants
Figure 25. Heatmap and cluster dendrogram of relative abundances of CLR-transformed samples

Samples are plotted left to right and ordered by the dendrogram. The dendrogram was generated from CLR-transformed read counts grouped by genera, based on the average linkage clustering of per-sample Aitchison distance. Branches of the dendrogram are coloured by sample type (stents are navy, urine are orange). The heatmap represents the relative abundance of genera within samples (more abundant genera are lighter in colour). Colour coding below the heat map corresponds to patient sex (females are pink, males are blue). An excerpt from the fourteen left-most branches of the tree illustrates that in general, samples from the same individual group nearby on the dendrogram.
A

Sample type
- Stent
- Urine

Sex
- F
- M

PC1: 11.7%
PC2: 8.8%

Female

PC1: 12.1%
PC2: 9.3%

Male

PC1: 11.5%
Figure 26. Principal component analysis of all samples

A PCA was performed on CLR-transformed Aitchison distances. Each coloured point represents a sample. Distance between samples on the plot represents differences in microbial community composition, with 20.5% of total variance being explained by the first two components shown. Strength and association for genera (sequence variants) are depicted by the length and direction of the gray arrows, respectively. Points are coloured by sample type (stents are navy, urine are orange); ellipses represent the 95% confidence interval of the sample types. All samples are shown in A) and shaped by participant sex (females are circles, males are triangles); female patients are shown in B), and males are shown in C).
A) Aitchison distance was greater between samples from different participants than within samples from the same participant (Bonferroni corrected Mann-Whitney U test, \( P < 0.0001 \)). B) Aitchison distance was greater from stent samples to urine within (W) the same participant than between proximal to distal stent curls of the same stent (Bonferroni corrected Mann-Whitney U test, \( P < 0.0001 \)). Aitchison distance was greatest between (B) urine of one participant to stent samples from the other participants (Bonferroni corrected Mann-Whitney U tests, \( P < 0.0001 \)). Box plot whiskers represent minimum and maximum.

**Figure 27. Microbiota similarity between samples assessed with beta diversity**

A) Aitchison distance was greater between samples from different participants than within samples from the same participant (Bonferroni corrected Mann-Whitney U test, \( P < 0.0001 \)). B) Aitchison distance was greater from stent samples to urine within (W) the same participant than between proximal to distal stent curls of the same stent (Bonferroni corrected Mann-Whitney U test, \( P < 0.0001 \)). Aitchison distance was greatest between (B) urine of one participant to stent samples from the other participants (Bonferroni corrected Mann-Whitney U tests, \( P < 0.0001 \)). Box plot whiskers represent minimum and maximum.
Figure 28. Microbial communities in longitudinally collected samples

Each vertical bar represents the relative SV abundance within a single sample. Samples are grouped by participant. Relative abundance of SVs is coloured by genera, with common genera shown in the legend. Sample and participant attributes are described in the legend and coloured accordingly (participant sex, grade of stent encrustation, sample type, and the visit number). Stents from the left side are denoted by “L” and from the right side by “R”, while urine are denoted by “U”. Days between sample collections are listed in the green visit code.
A) PCA was performed on CLR-transformed Aitchison distances of longitudinally collected samples. Each coloured point represents a sample. Distance between samples on the plot represents differences in microbial community composition, with 24.9% of total variance being explained by the first two components shown. Strength and association for genera (sequence variants) are depicted by the length and direction of the gray arrows, respectively. Points are coloured by participant and shaped by visit number.

B) Aitchison distance was greater between interindividual samples of the same type than between samples from the same participant of the same type at different visits (Mann-Whitney U test, P < 0.0001). Box plot whiskers represent minimum and maximum.

Figure 29. Principal component analysis of longitudinal samples
Figure 30. Microbial communities of bilateral stents

Each vertical bar represents the relative SV abundance within a single sample. Samples are grouped by participant. Relative abundance of SVs is coloured by genus, with common genera shown in the legend. Sample and participant attributes are described in the legend and coloured accordingly (participant sex, grade of stent encrustation, sample type). Stents from the left side are denoted by “L” and from the right side by “R”; Urine are denoted by “U”.
Figure 31. Principal component analysis of bilateral stents

A) PCA was performed on CLR-transformed Aitchison distances of samples from patients with bilateral indwelling stents. Each coloured point represents a sample. Distance between samples on the plot represents differences in microbial community composition, with 29.3% of total variance being explained by the first two components shown. Strength and association for genera (sequence variants) are depicted by the length and direction of the gray arrows, respectively. Points are coloured by participant and shaped by sample type. B) Aitchison distance was compared between interindividual and intraindividual samples. S = distance between stent samples from the same participant, U vs S = distance between urine and stent samples from the same participant, All = all samples from a single participant. All intraindividual comparisons had significantly shorter distances than the distance between samples from different individuals (Kruskall-Wallis test with Dunn’s multiple comparisons, P <0.0001). In intraindividual comparisons, the distance was shortest between stent samples and furthest from urine to stent samples (P = 0.022). Box plot whiskers represent minimum and maximum.
Table 15. Significant covariates of microbiota variation at genus level PCA ordination

<table>
<thead>
<tr>
<th>Metadata</th>
<th>P- value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stent indwelling time</td>
<td>0.008</td>
</tr>
<tr>
<td>Pulmonary disease</td>
<td>0.001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.001</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.001</td>
</tr>
<tr>
<td>IBS</td>
<td>0.002</td>
</tr>
<tr>
<td>Procedure (e.g. ureteroscopy, ESWL)</td>
<td>0.007</td>
</tr>
</tbody>
</table>
Table 16. Significant correlations between metadata attributes and the microbiota after adjusting for confounders

<table>
<thead>
<tr>
<th>Metadata</th>
<th>Genus</th>
<th>Coefficient</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Campylobacter</td>
<td>0.250</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td>Lactobacilli</td>
<td>-0.370</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Veillonella</td>
<td>0.457</td>
<td>0.002</td>
</tr>
<tr>
<td>BMI</td>
<td>Actinotignum</td>
<td>0.499</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td>Morganella</td>
<td>0.546</td>
<td>0.049</td>
</tr>
<tr>
<td>Stent indwelling time</td>
<td>Enterococcus</td>
<td>-0.292</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>Escherichia</td>
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</tr>
<tr>
<td></td>
<td>Finegoldia</td>
<td>0.202</td>
<td>0.034</td>
</tr>
<tr>
<td></td>
<td>Porphyromonas</td>
<td>0.245</td>
<td>0.001</td>
</tr>
<tr>
<td>Pulmonary disease</td>
<td>Campylobacter</td>
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<td>0.004</td>
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<td></td>
<td>Ezakiella</td>
<td>0.498</td>
<td>0.034</td>
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<td>Hypertension</td>
<td>Campylobacter</td>
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<td>0.013</td>
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<tr>
<td></td>
<td>Klebsiella</td>
<td>0.592</td>
<td>0.034</td>
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<td></td>
<td>Moryella</td>
<td>0.349</td>
<td>0.035</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Citrobacter</td>
<td>-1.653</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Enterococcus</td>
<td>1.086</td>
<td>0.034</td>
</tr>
<tr>
<td>IBS</td>
<td>Prevotella</td>
<td>0.046</td>
<td>0.001</td>
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<td></td>
<td>Veillonella</td>
<td>0.041</td>
<td>0.020</td>
</tr>
<tr>
<td>IBD Ulcerative Colitis</td>
<td>Lactobacillus</td>
<td>-0.112</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>Staphylococcus</td>
<td>0.009</td>
<td>0.061</td>
</tr>
<tr>
<td></td>
<td>Veillonella</td>
<td>0.036</td>
<td>0.020</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>Aerococcus</td>
<td>-0.424</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>Ureaplasma</td>
<td>-0.816</td>
<td>0.003</td>
</tr>
</tbody>
</table>
Table 17. Classification of stent encrustation

<table>
<thead>
<tr>
<th>Grade of encrustation</th>
<th>Visual characteristics</th>
<th>Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Like-new</td>
<td>84</td>
</tr>
<tr>
<td>1</td>
<td>Discolouration only</td>
<td>308</td>
</tr>
<tr>
<td>2</td>
<td>Mild encrustation (≤1 mm thick)</td>
<td>135</td>
</tr>
<tr>
<td>3</td>
<td>Heavy encrustation (&gt;1 mm thick)</td>
<td>10</td>
</tr>
</tbody>
</table>
Figure 32. Relationship of stent encrustation to indwelling time and alpha diversity

A) Indwelling time was positively correlated with the degree of stent encrustation, as calculated by the Spearman correlation coefficient ($r = 0.3221$, $P < 0.0001$). B) Shannon’s index of alpha diversity was negatively correlated with the degree of stent encrustation, as calculated by the Spearman correlation coefficient ($r = -0.1378$, $P = 0.0005$). C) Shannon’s index was lower for grade-3 encrusted stents compared to grade-0 (Kruskall-Wallis test with Dunn’s multiple comparisons, $P = 0.027$).
Figure 33. Microbial communities of long-term stents

Each vertical bar represents the relative SV abundance within a single sample. Samples are grouped by participant. Relative abundance of SVs is coloured by genus, with common genera shown in the legend. Sample and participant attributes are described in the legend and coloured accordingly (participant sex, grade of stent encrustation, sample type). Stents from the left side are denoted by “L” and from the right side by “R”, while urine are denoted by “U”. Stent indwelling time is stated under each participant (from 62 to 394 days).
Figure 34. Stent microbiota and encrustation are unchanged by antibiotics, device placement method, and UTI

PCA was performed on CLR-transformed Aitchison distances. Each coloured point represents a sample. Distance between samples on the plot represents differences in microbial community composition, with 20.5% of total variance being explained by the first two components shown. Strength and association for genera (sequence variants) are depicted by the length and direction of the gray arrows, respectively. Samples are coloured based on (A) whether the study participant had used antibiotics within the last 30 days prior to sample collection (blue) or not (pink), (D) whether the stents were placed in a retrograde (purple) or antegrade (orange) manner, and (G) whether the participant had a UTI within 7 days of stent placement or throughout the indwelling period (orange), or not (green). Ellipses represent the 95% confidence interval. (B, E, H) The degree of stent encrustation was compared between groups of interest. Groups were not significantly different by two-tailed Mann-Whitney U test. Shannon’s index of alpha diversity was not significantly different between antibiotic (C) or placement (F) groups, but patients with a UTI had lower diversity than those without (I, two-tailed Mann-Whitney U test, P = 0.002). Box plot whiskers represent minimum and maximum.
4.4.3 SEM imaging of stents

SEM imaging of stent samples revealed characteristic crystal phases and the presence of bacterial biofilms (Figure 35). Where bacteria-like structures were visualized, their morphology showed concordance which the genera that were present in the sample based on microbiota sequencing (Figure 35, samples 014 and 195). The predominant substances on the stent surfaces consisted of organic deposits and crystals. X-ray diffraction of crystalline structures confirmed the presence of calcium oxalate monohydrate in oval and multiple-twinning morphologies, calcium oxalate dihydrate, calcium phosphate (Figure 35), uric acid, and struvite (not shown).
Figure 35. Scanning electron micrographs of stents

Representative scanning electron micrographs of stent encrustations illustrating typical bacterial biofilms and crystal morphologies. Based on microbiota sequencing, bacteria visible (white arrowheads) likely correspond the genera (014) *Lactobacillus* and (195) *Enterococcus*. X-ray diffraction of crystalline microstructures (white arrows) correspond to calcium oxalate dihydrate (010 and 019a), calcium oxalate monohydrate in oval (022) and multiple-twinning (095) morphologies, and calcium phosphate (019b and 195). Scale bars = 20 µm.
4.5 Discussion

This study is the first to characterize the urinary microbiota of ureteral stent patients and the largest of its kind to evaluate the microbiota present on the surface of ureteral stents. Importantly, we identified several novel patient factors and comorbidities as drivers of stent microbiota composition and demonstrated that sex, antibiotic use, and stent placement method did not have a significant impact on the urinary or stent microbiota. Our findings also demonstrate consistency in stent microbial community over time in patients with multiple stent placements, and in both left and right sides during bilateral stent placement, solidifying the true presence of a reproducible stent microbiota.

Interestingly, intraindividual microbiotas of proximal and distal stent ends were more similar than either stent end compared to the urine, indicating that although the same genera may be present in the urine, it is not proportionally representative of the bacterial community colonizing the stent.

Previous culture-based studies have shown that removed stents are frequently culture positive despite patients exhibiting a culture-negative urine profile (Kehinde et al., 2004; Riedl et al., 1999). Corroborating these findings, we demonstrated that urinary and stent microbiotas were dominated by similar bacterial genera; however, when investigating the patterns on a per-patient basis, both proximal and distal ends of the stent, as well as left and right bilateral stents, were more similar to each other in microbiota composition than to the urine. Additionally, culture confirmed UTI was not associated with increased encrustation level in this cohort. This illustrates that urine is not an accurate biomarker of stent encrustation, nor representative of the stent-adhered microbiota. Instead, the degree of stent encrustation was positively correlated with indwelling time and negatively correlated with microbial diversity, indicating that the longer a stent is indwelling, the greater the likelihood of it becoming encrusted and colonized with a less diverse microbial community.

The urinary microbiome may extend as far as the renal collecting system. This renal microbiota may contribute to the microbial community of the proximal stent curl, or bacteria residing in the bladder could adhere to the proximal stent curl during retrograde
insertion (Barr-Beare et al., 2015; Tavichakorntrakool et al., 2017). Bacteria are also thought to ascend from the distal curl during movement of the stent whilst indwelling, or by utilizing active motility, a process that can occur quite rapidly (Chew et al., 2007; Lane et al., 2007; Nickel et al., 1992; Reid et al., 1992; Siitonen and Nurminen, 1992; Tenke et al., 2012). Our findings support these previous studies and suggest that the stent-associated microbiota is derived from the urinary bladder based on the fact that no difference was observed in microbial composition between antegrade or retrograde placement method (though only 9% of stents were inserted in an antegrade fashion). Further validation is provided by the stent-associated microbiota being dominated by common urinary bacteria which is unlikely to have originated from skin or gut contamination during placement (Gottschick et al., 2017). Taken together, our findings suggest that although the urinary microbiota may originally seed onto the stent, the stent microbial community is shaped and enriched for competitively adherent bacteria, and eventually diverges significantly from the urine.

A previous microbiota study of stent encrustations demonstrated a lack of association between “urotype” and patient conditions including age, gender, BMI, diabetes, urinary crystals, and other factors (Buhmann et al., 2019). The current study differed by utilizing a non-partisan analysis method, whereby arbitrary community groups, or “urotypes” were not used and instead the entire dataset was tested against all patient and sample characteristics. With this approach, confounders were adjusted for and significant associations between eight metadata features and genus-level microbiota changes were established.

In concordance with previous studies, age was determined to be significantly associated with decreased Lactobacillus spp. and increased Veillonella spp. (Liu et al., 2017; Rowe and Juthani-Mehta, 2013). In humans, Veillonella spp. are commensals of the oral cavity, gastrointestinal and urogenital tracts, with the potential to cause opportunistic infections, including UTI (Aujoulat et al., 2014; Berenger et al., 2015; Mashima and Nakazawa, 2014; Scheiman et al., 2019; van de Wijgert et al., 2014). Veillonella spp. are also commonly associated with a more diverse urinary microbiota, an attribute often accompanied with urological disorders (Pearce et al., 2015; Thomas-White et al., 2017).
In contrast, *Lactobacillus* spp. are commensals, with a robust body of evidence detailing their beneficial effects in the healthy urinary tract of both men and women (Aragon *et al*., 2018; Gottschick *et al*., 2017; Whiteside *et al*., 2015). It is unclear how the ageing process alters the urinary microbiota, however the observed decrease in protective urinary lactobacilli may account for common stent associated UTI and encrustation in older populations (Akay *et al*., 2007; Altunal *et al*., 2017).

Patients with IBS and IBD had increased stent and urinary presence of *Prevotella* and *Veillonella* species, and decreased lactobacilli. These findings are consistent with previous literature on the gut microbiota in these conditions (Lee and Tack, 2010; Sha *et al*., 2013; Shankar *et al*., 2013). These genera have also been implicated in urogenital infections and disorders such as pelvic inflammatory disease (Brook, 2004; Haggerty and Taylor, 2011). Our findings add further credence to the hypothesis that the gut microbiota is a reservoir for the genito-urinary microbiota (Magruder *et al*., 2019; Yamamoto *et al*., 1997). In the same manner that gut colonization with uropathogenic *Escherichia coli* (UPEC) increases the risk of UPEC UTI, the concurrence of inflammatory urinary tract symptoms in patients with IBS may be explained (Magruder *et al*., 2019; Matsumoto *et al*., 2012; Moreno *et al*., 2006; Moreno *et al*., 2008; Zingone *et al*., 2017). A limitation of the current study was that lower urinary tract symptoms were not evaluated in the stent patient population, but future studies should evaluate this if urological patients with IBS/IBD experience increased stent-associated complications in addition to the documented urinary tract symptoms.

Of the various comorbidities that significantly impacted the stent microbiota, it was notable that they originated from distant sites (pancreas, respiratory tract, liver, and gastrointestinal tract), suggesting some common physiological denominator. Potentially, it is the gastrointestinal tract that is altered by these conditions, with systemic consequences of bacterial translocation. For this reason, it is feasible that microbiota-based treatment including oral consumption of probiotic lactobacilli, or even fecal microbiota transplantation could be of therapeutic potential to stent patients. In addition to many other maladies, these treatments have shown efficacy against IBS/IBD.
symptoms, urogenital infections in the elderly, and recurrent UTI (Ducrotte et al., 2012; Hocquart et al., 2019; Kim and Park, 2017; Tariq et al., 2017).

The majority of stents imaged by SEM revealed encrustations composed of organic material and urinary crystals, while bacteria were only visualized in a small number of cases. This was expected due to the high proportion of urolithiasis patients amongst the study participants, as well as the low bacterial load present in urinary samples (Dyer and Nordin, 1967; Lewis et al., 2013). If these organisms were involved in crystal deposition on the biomaterial, the urologist should ensure device removal within 3 weeks, given the positive correlation between indwelling time and stent encrustation.

Due to the low bacterial biomass nature of the samples collected, this study utilized stringent pre-sequencing processing methods in addition to the application of conservative bioinformatic cut-offs and analysis tools in order to minimize contamination effects (Karstens et al., 2019; Minich et al., 2019). In future studies, quantification of total 16S rRNA gene copies by qPCR or the use of extended quantitative urine culture may complement and validate microbiota analysis of urinary samples (Hilt et al., 2014; Buhmann et al., 2019). Nevertheless, the detection of reproducible, patient-specific, stent microbiota signatures provides confidence that our findings are not due to contamination.

In summary, this study has characterized the urinary and stent microbiota of ureteral stent patients from a single centre over a one-year period, uncovering the importance of patient characteristics in explaining microbiota variation. Actions taken by the physician such as antibiotic treatment and stent placement method had little to no impact on the microbiota in these samples, but comorbidities and patient age did. The stent microbiota appears to originate from patient-specific adhesion of urinary microbes, and subsequently diverges to a distinct reproducible population, thereby negating the urine as an accurate biomarker for stent encrustation or microbiota status. These findings suggest that timely stent removal is likely the most important action to be taken by the treating urologist. Stent-specific antibiotic administration practices need recalibration, perhaps interchanging prophylactic oral antibiotic use with targeted intravesical antimicrobial instillation during device placement (Pietropaolo et al., 2018). Elderly patients or those diagnosed with
pulmonary disease, hypertension, diabetes, or IBS/IBD may need closer evaluation to minimize stent- and microbiota-associated complications.

4.6 References


stents from patients without urinary tract infection reveal distinct urotypes and a low bacterial load. Microbiome 7, 60.


syndrome: a large-scale internet survey in Japan using the overactive bladder symptom score and Rome III criteria. BJU Int. 111, 647-652.


Chapter 5

5 Utilization of a *Drosophila melanogaster* model of stone formation to explore host-microbe interactions in nephrolithiasis

5.1 Abstract

The prevalence of nephrolithiasis in North America has risen to approximately 10% and is associated with significant morbidity. The microbiota is known for its role in human health and disease, including in kidney stone formation. Intestinal colonization with *Oxalobacter formigenes* has been suggested to protect against formation of calcium oxalate (CaOx) stones by reducing urinary oxalate, and kidney stones are known to harbour uropathogenic bacteria. In stone patients, probiotic supplementation with oxalate-degrading bacteria has been suggested as a potential preventive therapy, but clinical trials with *O. formigenes* have been limited and inconclusive. The aims of this study were to investigate the oxalate-degrading properties of *Bacillus subtilis* 168 (BS168) as a potential probiotic candidate and to characterize how uropathogens may be contributing to stone formation using an established *Drosophila melanogaster* (DM) model of urolithiasis and *in vitro* cell line experiments. Flies administered BS168 developed lower fecal and Malpighian tubule crystal burden compared to flies without bacterial treatment and had increased survival. Uropathogenic *Escherichia coli* strain UTI89 increased stone burden but did not impact DM survival, whereas *Proteus mirabilis* PM175 decreased survival irrespective of lithogenesis. *In vitro* cell line experiments with BS168 did not exhibit increased crystal aggregation, suggesting this strain is a promising probiotic candidate to reduce stone formation through degradation of oxalate. Conversely, uropathogenic *E. coli* but not *P. mirabilis* may increase stone burden through its capacity to increase CaOx crystal adhesion and aggregation. As strains of *B. subtilis* have been used safely to promote digestive health, strain BS168 is worthy of testing in humans to determine if it can reduce the incidence of recurrent nephrolithiasis.
5.2 Introduction

The prevalence of nephrolithiasis, or kidney stone disease, in North America has risen to approximately 10% over recent decades. The condition is associated with significant patient morbidity and severe economic losses to the health care system. The human microbiota, defined as the microorganisms present in a certain environment, is recognized as an important facet in health and disease, including nephrolithiasis for several reasons (Whiteside et al., 2015). One reason involves the gut microbiota’s capacity to influence serum oxalate levels, a terminal metabolite implicated in approximately 80% of all kidney stones (Moe, 2006). Another reason deals with the urinary microbiota and how it may interact with stone formation at the site (Barr-Beare et al., 2015).

The most common kidney stones are composed of calcium oxalate, of which oxalate is the limiting factor (Moe, 2006). Urinary oxalate is derived from both dietary and endogenous sources, where estimates of the relative contributions vary widely; studies have suggested that the proportion of dietary derived urinary oxalate ranges from 10-50% (Taylor and Curhan, 2008; Holmes et al., 2001). Dietary oxalate can be absorbed by both trans- and paracellular mechanisms in its soluble form, excreted in the feces as an insoluble crystal, or degraded by members of the gut microbiota. The bacterium Oxalobacter formigenes utilizes oxalate as its sole carbon source, and when present in the intestine, subjects have been reported to have lower urinary oxalate levels and are subsequently at lower risk of developing stones (Kaufman et al., 2008; Jiang et al., 2011). Other members of the gut microbial community including lactobacilli and bifidobacteria are capable of degrading oxalate, though to a lesser extent (Miller and Dearing, 2013). In kidney stone patients, supplementation with oxalate-degrading bacteria has been suggested as a potential preventive therapy, however trials thus far have been limited and inconclusive (Hoppe et al., 2006; Siener et al., 2013; Klimesova et al., 2014).

As with O. formigenes, Bacillus subtilis strain 168 (BS168) can degrade oxalate (Tanner and Bornemann, 2000). The strain’s oxalate decarboxylase gene, YvrK, is acid-induced and encodes a ~43 kD manganese-requiring enzyme that converts oxalate to formate and
CO₂ (Tanner et al., 2001). Some *Bacillus subtilis* strains have recently been identified for potential probiotic use for gastrointestinal disorders and have the added benefit of being highly heat and pH resistant due to spore formation (Sorokulova et al., 2008; Poormontaseri et al., 2017). It is for these reasons that BS168 may have therapeutic potential in nephrolithiasis treatment.

Struvite stones have long been known to be associated with urinary tract infection by urease-producing organisms such as *Proteus, Pseudomonas*, or *Klebsiella* spp., whereas other stone compositions have historically not been thought to involve a bacterial component (Flannigan et al., 2014). However, recent culture-dependent and -independent studies have identified the presence of bacteria within non-struvite kidney stones (Barr-Beare et al., 2015; Dornbier et al., 2019; Golechha and Solanki, 2001; Sohshang et al., 2000; Tavichakorntrakool et al., 2012; Wang et al., 2014; Zampini et al., 2019). Whether these bacteria are coincidentally entrapped in the growing stone matrix, or if they are playing an active role in stone formation remains to be elucidated.

In this study, the aim was to investigate the role of both pathogenic and non-pathogenic bacteria in nephrolithiasis. An established *Drosophila melanogaster* (DM) model of stone formation was used to evaluate stone burden after supplementation with both BS168 and uropathogenic bacteria (Chen et al., 2011). In addition, Madin-Darby renal epithelial cell culture was used to determine the impact of the microbes on calcium oxalate crystal adhesion and aggregation. Furthermore, to better assess the impact of various treatments on stone disease in the DM model, method development of an X-ray micro computed tomography (µCT) imaging protocol was undertaken. It was hypothesized that based on its safe nature and oxalate-degrading ability, that BS168 would reduce stone burden and promote markers of health, in contrast to uropathogens.

### 5.3 Materials and Methods

#### 5.3.1 Bacterial culture and growth curves

*Bacillus subtilis* strain 168 (ATCC 23857, designated here as BS168), *Escherichia coli* UTI89 (Mulvey et al., 2001) and a clinical kidney stone isolate of *Proteus mirabilis* (referred to here as PM175, Table 10), were routinely cultured at 37 °C in Luria-Bertani
(LB) broth (Table 18). BS168 growth in oxalate was assayed in 96-well plates prepared as dilutions of sodium oxalate (NaOx) in LB broth with stationary-phase bacteria added at a final dilution of 1/100. Plates were incubated for 24 hours at 37 °C with optical density (OD) readings every 30 minutes using an Eon microplate spectrophotometer (BioTek, Winooski, VT, USA). NaOx concentrations were selected based on physiologic intestinal oxalate concentrations reported in the literature (Prokopovich et al., 2007). Statistical analyses of growth curves were performed with the R package Growthcurver (Sprouffske and Wagner, 2016).

Bacteria were cultured from whole DM after homogenization. Fruit flies were surface sterilized with 70% ethanol and homogenized in 0.01 M PBS using a motorized pestle. Homogenates were then serially diluted and plated onto agar. Based on the known microbiota of DM, LB, MRS, and Mannitol agars were used to culture BS168, *Lactobacillus* spp., and *Acetobacter* spp., respectively (Table 18). LB and Mannitol plates were incubated aerobically at 37 °C, and MRS plates were incubated using an anaerobic gas pack (BD, Mississauga, ON, CAN) in a sealed jar at 37 °C. Colony forming units (CFU) were enumerated after 48-hours of incubation. Variation was observed between biological replicates, so oxalate-fed fly CFU counts were normalized to control diet-fed fly CFU counts.
## Table 18. Bacterial media compositions

<table>
<thead>
<tr>
<th>Media</th>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Luria-Bertani</strong></td>
<td>DI water</td>
<td>1000 mL</td>
</tr>
<tr>
<td></td>
<td>Tryptone</td>
<td>10 g</td>
</tr>
<tr>
<td></td>
<td>NaCl</td>
<td>10 g</td>
</tr>
<tr>
<td></td>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td></td>
<td>Agar (omit for broth)</td>
<td>15 g</td>
</tr>
<tr>
<td><strong>MRS agar</strong></td>
<td>DI water</td>
<td>1000 mL</td>
</tr>
<tr>
<td></td>
<td>Difco MRS Lactobacilli broth (BD)</td>
<td>55 g</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>15 g</td>
</tr>
<tr>
<td><strong>Mannitol agar</strong></td>
<td>DI water</td>
<td>1000 mL</td>
</tr>
<tr>
<td></td>
<td>Mannitol</td>
<td>25 g</td>
</tr>
<tr>
<td></td>
<td>Yeast extract</td>
<td>5 g</td>
</tr>
<tr>
<td></td>
<td>Peptone</td>
<td>3 g</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>15 g</td>
</tr>
</tbody>
</table>
5.3.2 *Drosophila melanogaster* husbandry

As insects, the use of *Drosophila melanogaster* (DM) in this study did not require institutional ethical review board approval. The DM stock used in this study was Canton-S. Standard cornmeal-based media was utilized (Table 19), and the lithogenic diet included 0.1 or 1.0 % weight/volume NaOx (Chen *et al*., 2011). Food was generated by gently boiling the water on a hot plate, and slowly adding all components except the acid mix and NaOx (if including), being sure to bring to the boil in between additions. The food was then cooled to 55 °C, at which point the acid mix and oxalate were added and mixed well. The media was portioned to approximately 50 mL per bottle or 10 mL per vial and stored at 4 °C until use. Grape juice agar was made by boiling the water, agar, and juice in a microwave, cooling to 55 °C at which point the Tegosept was added and the solution was poured into Petri dishes. DM were maintained in a 25 °C incubator with 60% humidity and a 12 h light-dark cycle. For maintenance, DM stocks were transferred to fresh 50 mL media bottles every two weeks; for experiments, DM were transferred to fresh 10 mL media vials every two days. Lifespan measurement was performed as previously described (Linford *et al*, 2013).
Table 19. *Drosophila melanogaster* media and chemicals

<table>
<thead>
<tr>
<th>Recipe</th>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard media</td>
<td>DI water</td>
<td>1000 mL</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>15 g</td>
</tr>
<tr>
<td></td>
<td>Dried yeast</td>
<td>17.3 g</td>
</tr>
<tr>
<td></td>
<td>Cornmeal</td>
<td>73 g</td>
</tr>
<tr>
<td></td>
<td>Corn syrup</td>
<td>76 mL</td>
</tr>
<tr>
<td></td>
<td>Acid mix (50 mL propionic acid with 3.2 mL phosphoric acid)</td>
<td>5.8 mL</td>
</tr>
<tr>
<td>Grape juice agar</td>
<td>DI water</td>
<td>750 mL</td>
</tr>
<tr>
<td></td>
<td>Agar</td>
<td>30 g</td>
</tr>
<tr>
<td></td>
<td>Welch’s grape juice</td>
<td>250 mL</td>
</tr>
<tr>
<td></td>
<td>Tegosept (10 % solution in ethanol)</td>
<td>10 mL</td>
</tr>
<tr>
<td>Yeast paste</td>
<td>DI water</td>
<td>6 mL</td>
</tr>
<tr>
<td></td>
<td>Dried yeast</td>
<td>3 g</td>
</tr>
<tr>
<td>Carl’s solution</td>
<td>DI water</td>
<td>28 mL</td>
</tr>
<tr>
<td></td>
<td>95% ethanol</td>
<td>17 mL</td>
</tr>
<tr>
<td></td>
<td>Formalin</td>
<td>6 mL</td>
</tr>
<tr>
<td></td>
<td>Glacial acetic acid</td>
<td>2 mL</td>
</tr>
</tbody>
</table>
5.3.3 Determining the effect of bacterial supplementation in the DM stone model

An experimental timeline is displayed in Figure 36. At five days of age, adult DM were sorted into cohorts and supplemented with 5% sucrose solution with or without bacteria for 24 hours. Bacteria were prepared for supplementation to DM as follows: 25 mL overnight broth culture of BS168, UTI89, or PM175 (~ $10^8$ CFU/mL) was pelleted by centrifugation at 5000 rpm for 5 minutes. The supernatant was decanted, and the pellet was completely resuspended in 25 mL sterile PBS by vortexing. The process was repeated whereby the PBS and BS168 was centrifuged at 5000 rpm for 5 minutes, after the supernatant was decanted, and the pellet was resuspended in 25 mL sterile PBS for a total of two PBS washes. The remaining pellet after the second PBS wash was completely resuspended in 25 mL of 5% weight/volume sucrose solution by vortexing. DM were transferred to polypropylene vials containing a cotton ball moistened with 3 mL of 5% sucrose ± bacteria. After 24 hours, DM were transferred to standard media ± 1.0% NaOx for BS168, or 0.1% NaOx for the uropathogen experiments.

For larval exposure to bacteria, on Day 0 approximately 200 adult DM were mated in standard media bottles containing 1 cm x 1 cm x 0.25 cm grape juice agar and 1 cm diameter of yeast paste (water and yeast mixed to the consistency of smooth peanut butter) (Table 19). DM were allowed to lay eggs for 3 hours, after which all adults were removed along with the grape agar and any remaining yeast paste. Five hundred µL of 5% sucrose ± bacteria (processed as above) was then pipetted on the top of the standard media. On Day 2, 100 mL of room temperature 20% sucrose solution was added to completely submerge the media in the bottles and float the larvae for 20 minutes. The sucrose solution containing larvae was then gently poured over a sterile cell strainer. The larvae collected in the strainer were briefly rinsed once with 70% ethanol, then twice with DI water. Cleaned larvae were added with a paintbrush to vials of standard media ± 1.0% NaOx.

Stone burden was evaluated in adult DM Malpighian tubules on day 7. Briefly, DM were narcotized with CO$_2$, treated for 2 minutes in Carl’s solution (Table 19) in a small glass petri dish, and transferred to a dissecting dish with cold sterile PBS. Using a Nikon
SMZ800N stereomicroscope, DM Malpighian tubules were dissected in the cold PBS, fixed in 4% formaldehyde/PBS for one hour at room temperature, and mounted on microscope slides in 50:50 PBS and glycerol. Clear nail polish was used to seal the coverslip to the microscope slides prior to polarized light microscopy. Birefringence microscopy of the dissected tubules was performed with a Nikon Ts2R inverted microscope, and NIH ImageJ software was utilized to determine the degree of birefringence per tubule (particle analysis function). Fecal excreta from adult DM was also evaluated for the presence of birefringent crystals throughout the duration of the 14-day BS168 survival analysis, as previously described, Figure 37 (Ali et al., 2018). Briefly, glass cover slips were inserted into the fly vial plugs during the 14-day incubation period, then fixed to microscope slides with clear nail polish and imaged with polarized light (Nikon) to determine the percentage of excreta containing birefringent crystals.

Stone burden was evaluated in third instar DM larvae on day 4 by means of a crawling assay; larval crawling is an indicator of behavioural and locomotor health in DM (Nichols et al., 2012; Gunther et al., 2016). Twenty mL of room temperature 20% sucrose solution was added to each media bottle for 20 minutes, during which larvae floated to the top. The solution was then poured over a sterile cell strainer, where the collected larvae were washed twice with DI water. A paint brush was used to transfer larvae from the strainer to a 15 mL petri dish containing 2% agar where they acclimatized for 10 minutes. A second petri dish of 2% agar was positioned over a 0.5 cm graph paper grid (Figure 37). A six mm diameter paper disk was submerged in apple cider vinegar and positioned near one side of the plate, and larvae were positioned at a starting point 4 cm away. The time it took each larva to travel 3 cm from the starting point towards the vinegar stimulant was recorded.
Figure 36. *Drosophila melanogaster* experimental timeline

Schematic outline of experimental design. A) Adults were supplemented +/- BS168, UTI89, or PM175 in 3 mL of 5% sucrose via cotton balls in empty vials on Day 0. On Day 1 they were transferred to standard media vials with or without 1.0% or 0.1% NaOx (weight/volume). Deaths were recorded daily, and dead flies were removed every two days during transfer to fresh food. Fecal excreta were assayed throughout the 14-day BS168 experiment by transferring the coverslip-embedded vial plug with each food transfer. Lifespan analysis of DM uropathogen experiments was conducted until the expiration of all DM (approximately 60 days). B) Adult DM mated for three hours in standard media bottles, after which they were removed and 500 uL of 5% sucrose +/- BS168, UTI89, or PM175 was pipetted over the top of the media. They were transferred via 20% sucrose suspension to standard media +/- 1.0% NaOx on Day 2 and their crawling was assayed on Day 4.
Figure 37. Assays of stone burden and DM health

A) Fecal excreta assay. Microscope coverslips remained inserted in the vial plug throughout the duration of the 14-day adult DM survival experiment and were evaluated for crystal birefringence with polarized light microscopy. B) Larval locomotion assay. Larvae were stimulated to crawl towards the apple cider vinegar attractant; the time taken to travel 6 squares (3 cm) was recorded.
5.3.4 Development of a µCT scanning protocol in DM

As human kidney stones are normally assessed with computed tomography (CT) imaging, we sought to develop a mechanism of live imaging DM for the purpose of evaluating stone burden, in collaboration with the Robarts Research Institute Micro-CT lab (Assimos et al., 2016; Poinapen et al., 2017). A custom scanning apparatus was designed to house flies throughout the duration of scanning (Figure 38). Flies were imaged with a constant flow of CO$_2$ anesthesia (0.5 mL/min) and X-ray radiation (90 kVp; 70 uA; 0.3 degree incremental angle over 360 degrees) using the GE Locus MS scanner (GE Healthcare, Mississauga, ON, CAN). Image analysis was performed using a threshold of 700 Hounsfield units (HU) as the minimum density of stone particles (Shahnani et al., 2014). Particles greater than 10 voxels in size were retained for stone quantification analysis. Adult flies treated with BS168 and 1.0% NaOx as described above were imaged on Day 3 and Day 7.
A) Acrylic apparatus developed for live µCT imaging of anesthetized DM. The DM chamber (B) was mounted on the rotating base inside the CO₂ chamber. Four DM were placed into wells on each of two foam scanning platforms, separated by cohort. Desiccation was prevented by the addition of hydrated foam, and a water-filled capillary was utilized for scan calibration.

Figure 38. DM µCT scanning apparatus
5.3.5  CaOx crystal adhesion to renal epithelial tissue culture

Cell culture experiments were performed in Madin-Darby canine kidney (MDCK) epithelial cells (Bigelow et al., 1998; Yamaguchi et al., 2002), which were initially isolated from the normal renal tubules of an adult male cocker spaniel dog (Gaush et al., 1966), and were acquired from the American Type Culture Collection (ATCC). Cells were maintained in T75 flasks in a 5% CO₂ tissue culture incubator with minimum essential medium (MEM; Gibco, Burlington, ON, CAN) supplemented with 10% fetal bovine serum (FBS; Gibco) and 2 mM L-Glutamine (Gibco), the combination of which is hereafter referred to as MEM. Cells were routinely passed when confluency reached 80% and above. To pass the cells, the spent MEM was removed from the flask and cells were washed with warmed phosphate buffered saline (PBS), followed by trypsinization for approximately 10 minutes at 37 °C and 5% CO₂ by addition of 0.25% trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA; Gibco). After 10 minutes the cells were observed under the microscope for 90% detachment, when MEM was added to inactivate the trypsin. For cell maintenance, cell suspension was seeded to new flasks at a subcultivation ratio of 1:6 with fresh MEM. For experiments, an aliquot of the cell suspension was mixed 1:1 (v/v) with 0.4% trypan blue (Invitrogen, Carlsbad, CA, USA) and 10 μL of sample mixture added to the chamber of a Countess Cell Counting Chamber Slide (Invitrogen) for use with a Countess II Automated Cell Counter (Invitrogen). Cell suspensions were centrifuged at 2,000 rpm for 5 minutes, reconstituted in culture media, and subsequently seeded to 6-well tissue-culture treated plates (Sarstedt, Nümbrecht, Germany) at 1 x 10^5 cells/mL. Plates were incubated as above until the monolayer reached confluency, at approximately 48 hours.

Utilizing a method adapted from Bigelow et al., calcium oxalate monohydrate (COM) crystal adhesion and aggregation was investigated in the MDCK cells (Bigelow et al., 1998; Yamaguchi et al., 2002). An overnight broth culture of BS168, UTI89, or PM175 was processed as described earlier but the bacterial pellet was reconstituted in MEM at a concentration of ~5 x 10^3 CFU per mL. Confluent monolayers of MDCK cells in 6-well plates were washed twice with warmed sterile PBS, then incubated with 2 mL MEM ± bacteria for 20 minutes at 37 °C and 5% CO₂. Cells were then washed twice with warmed
sterile PBS and incubated with 2 mL of artificial urine ± 0.5 mg/mL COM for 20 minutes at 37 °C and 5% CO₂ (Brooks and Keevil, 1997). The urine was then removed, and cells were gently washed with PBS. Two mL MEM was added, and cells were immediately imaged with light microscopy using a Nikon Ts2R inverted microscope. Crystal attachment was quantitated with ImageJ.

Statistical analysis for DM and cell culture experiments was conducted with GraphPad Prism (version 8.1.2) for Mac OSX (GraphPad Software, San Diego CA, USA). Results were considered significant as follows: ****, P<0.0001; ***, P< 0.001; **, P < 0.01; *, P < 0.05.

5.3.6 16S rRNA gene sequencing

Analysis of the adult DM microbiota was determined by 16S rRNA gene sequencing of 10 individual flies per cohort on Day 7. Specifically, five sex-separated vials were prepared per treatment group containing approximately 20 flies, and one fly (either male or female) was used from each. DNA was extracted from the single whole flies in accordance with the Earth Microbiome Project standard protocols, using the Qiagen DNeasy PowerSoil 96-well kit (Qiagen, Toronto, ON, CAN). A Biomek 3000 laboratory automation workstation (Beckman-Coulter, Mississauga, ON, CAN) was utilized for PCR reagent set up. Amplifications of the V4 region of the 16S ribosomal RNA gene were carried out with the primers (5’-3’)

ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNN……GGTGCAGCMGCCGCGGTAA and (5’-3’) CGGTCTCGGATTCCTGCTGAACCGCTCTTCCGATCTNNNN……GGACTACHVGGGTWTCTAAT wherein ccccccccccc is a nucleotide barcode and the preceding sequence is a portion of the Illumina adapter sequence for library construction (Parada et al., 2016). Two μL of DNA template was added to a 96-well plate containing 10 μL of both forward and reverse primers (3.2 pMole/μL) where unique barcode pairs were utilized for each sample. 20 μL of Promega GoTaq® Colourless Master Mix (Promega, Maddison, WI) was added to the DNA template and primers. The final plate was firmly sealed with a foil PCR plate cover. This plate was placed in the Eppendorf Mastercycler® thermal cycler (Eppendorf, Mississauga, ON), where the lid was kept at 105 °C. An initial warm-up temperature of
95 °C was used for 2 min to activate the GoTaq®. Afterwards, the volumes underwent 25 cycles of 95 °C for 1 min, 52 °C for 1 min, and 72 °C for 1 min. After completion, the temperature of the thermal cycler was held at 4 °C, and amplicons were then stored at −20 °C.

Processing of DNA samples and DNA sequencing was conducted at the London Regional Genomics Centre at Robarts Research Institute (London, ON). Amplicons were quantified using pico green (Quant-It; Life Technologies, Burlington, ON) and pooled at equimolar concentrations before cleanup (QIAquick PCR clean up; Qiagen, Germantown, MD). The final samples were sequenced using the MiSeq by Illumina® platform, with 2 × 260 bp paired-end chemistry. The returned reads were then analyzed using R, DADA2, the SILVA database (version 132), and ALDEx2 (R Core Team, 2019; Callahan et al., 2016; Quast et al., 2013; Fernandez et al., 2013).

5.3.7 qPCR-based quantification of microbial communities in DM

DNA template from the Qiagen DNeasy PowerSoil kit was also utilized for qPCR-based quantification. Bacterial loads were determined by qPCR using the Power SYBR Green kit (Applied Biosystems) following the manufacturer’s instructions. Universal 16S rRNA gene, genus-, and species-specific primer sets used in this study are listed in Table 20. All qPCR reactions were performed in DNase- and RNase-free 384-well microplates on a Quant Studio 5 Real-Time PCR System (Applied Biosystems) and analyzed with associated software. Copy numbers of target 16S rRNA genes were calculated as previously described using established primer efficiencies and limits of detection (Daisley et al., 2019; Walker et al., 2017).
<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5’&gt;3’)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host endogenous control</td>
<td>DRO_B-act_F</td>
<td>GGAAACCACGCAAATTTCAGT</td>
<td>Elgart et al., 2016</td>
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<td></td>
<td>DRO_B-act_R</td>
<td>CGACAACCAGAGCAACTTT</td>
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<tr>
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<td>CCGAAAAGTCTTGCTTTGTTCT</td>
<td>Daisley et al., 2017</td>
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<td></td>
<td>DRO_RpLP0_R</td>
<td>CGCTGCCTTTGCTCCCTAA</td>
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<tr>
<td>Universal bacteria</td>
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<td>Hartman et al., 2009</td>
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<tr>
<td></td>
<td>BAC_UNI_R</td>
<td>ATTACCGCGGTGCTGCTGTC</td>
<td></td>
</tr>
<tr>
<td>Acetobacter spp.</td>
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<td>TAGTGGCGGACGGGTGAGTA</td>
<td>Elgart et al., 2016</td>
</tr>
<tr>
<td></td>
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<tr>
<td>Lactobacillus spp.</td>
<td>BAC_Lacto_F</td>
<td>AGGTAACGGCTACCATGGC</td>
<td>Elgart et al., 2016</td>
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<td></td>
<td>BAC_Lacto_R</td>
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<td>CTCAGGTGCTGCTACGCACTG</td>
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5.4 Results

5.4.1 Increasing concentration of oxalate promotes the growth of BS168

As oxalate can be toxic to bacteria, even those capable of degrading it, the viability of BS168 was assessed upon exposure to oxalate concentrations ranging from 50 μM to 50 mM (Suryavanshi et al., 2016). Representative growth curves are presented in Figure 39. There was a significant, dose-dependent increase in growth when BS168 was supplemented with NaOx (Figure 39D).

5.4.2 Effect of dietary oxalate on culturable DM microbiota

DM reared on 0.1% or 1.0% w/v NaOx-containing media for seven days harboured significantly less (two-way ANOVA with Tukey’s multiple comparisons test, $P < 0.0001$) culturable bacteria compared to normal food controls (Figure 40). NaOx had a dose-dependent effect towards DM microbes cultured on LB and the *Acetobacter* spp. cultivated on Mannitol agar ($P = 0.0008$ and $P = 0.0049$, respectively). BS168 was detected by culture on LB agar from pulverized adult DM up to 5 days following supplementation (data not shown).
Figure 39. Increasing concentration of oxalate promotes the growth of BS168

A) Growth is represented as the increase in optical density at 600 nm over the course of 24 hours. BS168 was grown in LB broth with NaOx at the stated concentrations. Three biological replicates were performed; the mean of 10 representative replicates and SD are plotted. B) Area under the logistic curve from A), $n = 10$. C) Time at curve inflection from A), $n = 10$. D) Significant NaOx concentration comparisons between BS168 growth by Kruskall-Wallis test with Dunn’s multiple comparisons. All other comparison pairs were not significant.
Adult DM consumed normal food, or media with 0.1% or 1.0% (weight/volume) of NaOx for 7 days. DM were homogenized and plated onto Luria-Bertani (LB), de Man, Rogosa, Sharpe (MRS), or Mannitol agars. CFU from lithogenic groups were normalized to CFU from normal food groups to simplify variation from biological replicates. Average CFU/ fly for normal food cohorts was $2 \times 10^2$, $4 \times 10^4$, and $5 \times 10^4$ for LB, MRS, and Mannitol agars, respectively. Three biological replicates were performed, n = 15. Significance was determined with Tukey’s multiple comparisons test. Box plots illustrate the median, quartiles, minimum and maximum. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

**Figure 40. Lithogenic diet significantly depletes the culturable DM microbiota**

Adult DM consumed normal food, or media with 0.1% or 1.0% (weight/volume) of NaOx for 7 days. DM were homogenized and plated onto Luria-Bertani (LB), de Man, Rogosa, Sharpe (MRS), or Mannitol agars. CFU from lithogenic groups were normalized to CFU from normal food groups to simplify variation from biological replicates. Average CFU/ fly for normal food cohorts was $2 \times 10^2$, $4 \times 10^4$, and $5 \times 10^4$ for LB, MRS, and Mannitol agars, respectively. Three biological replicates were performed, n = 15. Significance was determined with Tukey’s multiple comparisons test. Box plots illustrate the median, quartiles, minimum and maximum. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.  

![Figure 40](image-url)
5.4.3 Effect of BS168 on stone burden in DM

Stone burden and health were assayed in the DM model of urolithiasis after supplementation with NaOx and BS168; adult survival, adult Malpighian tubule crystal birefringence, adult fecal excreta birefringence, and larval locomotion were all evaluated (Figure 41). Kaplan-Meier survival analysis of adult DM (Figure 41A) demonstrated that the detrimental effects of the highly lithogenic 1% NaOx diet were improved with supplementation of BS168 (logrank test, $P = 0.0057$). Larval crawling was significantly increased in 4-day old larvae treated with BS168 on lithogenic media when compared to untreated controls (Parametric, D’Agostino-Pearson test; vs. normal media controls, two-way ANOVA with Tukey’s multiple comparisons, $P = 0.0073$; vs. lithogenic controls, two-way ANOVA with Tukey’s multiple comparisons, $P = 0.0013$) (Figure 41B). On day 7, dissected adult Malpighian tubules (Figure 41C) from BS168-treated DM had significantly less CaOx crystal deposition compared to untreated lithogenic controls (Nonparametric, D’Agostino-Pearson test; Wilcoxon rank-sum test, $P < 0.0001$) (Figure 41D). The percentage of fecal excreta containing birefringent particles from adult DM vials after 14 days was significantly reduced in the BS168-treated cohorts relative to untreated lithogenic controls (Nonparametric, D’Agostino-Pearson test; Wilcoxon rank-sum test, $P = 0.0039$) (Figure 41E).
**A** Percent survival over days for different treatment groups:
- Control
- Control + BS168
- 1% Ox
- 1% Ox + BS168

**B** Box plot showing time to reach 3 cm (s) for different treatment groups. Significant differences are indicated by **.**

**C** Light microscopy and birefringence microscopy images:
- 1% Ox
- 1% Ox + BS168

**D** Birefringent pixels per MT (μm).

**E** Fraction birefringent excreta.

**F** Relative proportion of different genera across different treatment groups.

**Genera**
- Bacteroides
- Klebsiella
- Escherichia/Shigella
- Enterococcus
- Gilliamella
- Acetobacter
- Lactobacillus
**Figure 41. Effect of BS168 on DM model of urolithiasis**

A) Kaplan Meier survival analysis of five-day old DM that were supplemented with 2 \( \times 10^8 \) CFU of BS168 on Day 0, then transferred to normal or lithogenic media on Day 1 and followed for 14 days. Error bars represent SE. \( n \geq 100 \) per group from 4 biological replicates of \( n = 20-30 \) flies. B) Larval locomotion was determined on Day 4 after larvae were supplemented with 0.5 \( \times 10^8 \) CFU of BS168 on Day 0 and transferred to normal or lithogenic media on Day 2, \( n = 19-24 \) larvae per group from 3 biological replicates. C) I-IV) CaOx crystals were imaged in dissected Malpighian tubules from adult DM on Day 7 and quantitated with ImageJ particle analysis, \( n = 21-29 \) adults per group from 3 biological replicates. (D). C) V) Birefringent fecal excreta from coverslips in adult vials on Day 14 were imaged and the fraction that contained birefringent crystals was quantitated, \( n = 16-20 \) cover slips per group from 3 biological replicates. (E). Legend for group colours in A) is relevant for figures B), D), and E). Scale bar in C) is relevant for I-IV. F) Microbial composition of adult urolithiasis DM model exposed to BS168. DNA was extracted from whole adult DM after 7 days consuming normal or lithogenic media following ± BS168 supplementation on day 0. 16S rDNA was sequenced using the Illumina platform. Each column represents a single fly and each colour corresponds to a different bacterial genus. Five females and five males, each from separate vials, were included in each treatment group. Reads corresponding to *Wolbachia* were removed. All box plots illustrate the median, quartiles, minimum and maximum. ** \( P < 0.01 \), **** \( P < 0.0001 \).
5.4.4 16S rRNA gene sequencing assessment of DM microbiota

Microbial composition of whole pulverized adult DM was assessed after 7 days on normal or lithogenic media following BS168 treatment on Day 0. A common insect endosymbiont, *Wolbachia*, dominated the sequencing depth of all samples but was not significantly different between cohorts (Clark *et al*., 2005). Although *Wolbachia* is known to impact fertility, viral infection susceptibility, and longevity among other traits in DM, the bacterium is not known to play a role in the DM urolithiasis model, so reads corresponding to the genus were removed from downstream analysis (Clark *et al*., 2005; Teixeira, *et al*., 2008; Fry and Rand, 2002).

After omitting sequencing control samples (based on their distinct clustering apart from DM samples, Figure 42) the DM microbiota dataset contained 1,974,659 total reads, ranging from 10,945 to 76,733 reads across the 40 samples. An average of 2.72% of reads were removed from each sample following quality filtration performed utilizing the DADA2 pipeline (Callahan, *et al*., 2017). The remaining filtered 1,922,688 reads were assigned taxonomy with the SILVA (v132) training set. After filtering sequence variants to maintain those present at >1% in any sample, 69 sequence variants remained.

A bar plot representing the relative proportions of genera in the samples is represented in Figure 41F. These results are consistent with past surveys of the DM microbiota which exhibit a distinct and low-diversity microbiota dominated by the genera *Lactobacillus* and *Acetobacter*, which was observed here in DM consuming normal media (Wong *et al*., 2011). In the lithogenic diet cohorts the relative proportion of sequence variant 127, which based on sequence homology likely corresponds to *Acetobacter tropicalis*, was significantly decreased relative to normal media controls (Benjamini-Hochberg corrected Wilcoxon rank sum test, P = 0.0075), while lactobacilli proportions were unchanged. There were no differences in abundance of any bacterial groups when comparing BS168-treated with -untreated groups. No sequences corresponding to the genus *Bacillus* were detected from the flies in any cohort.

The sequence counts were centred log ratio (CLR) transformed, generating sample-wise Aitchison distances which were subsequently used to perform a principal component
analysis (PCA) (Figure 43A). Principal components 1 and 2 were plotted and represent 36.8% of the total variance in the data (Figure 43A). Samples did not partition into distinct groups based on treatment (coloured points), however subtle drivers in the data separation across principal component 1 were noted for diet groups and denoted with 95% confidence ellipses. Sequence variants driving separation of the dataset are depicted by the dark gray arrows and associated taxonomy. Differences in diversity metrics (Shannon’s Index of alpha diversity, Figure 43B, and Aitchison distance determination, Figure 43C) due to exposure to the lithogenic diet were observed in the un-supplemented controls but were mitigated when DM were supplemented with BS168.
Principal Component Analysis (PCA) plot of sequencing control and adult DM samples. A PCA was performed on CLR-transformed Aitchison distances. Distance between samples on the plot represents differences in microbial community composition, with 34.9% of total variance being explained by the first two components shown. DM samples are coloured by treatment groups, and sequencing control samples are red. The black ellipse represents the 95% confidence interval of the DM samples. Sequence variants are depicted by the gray numbers.

Figure 42. Sequencing controls distinctly separate from DM microbiota

Principal Component Analysis (PCA) plot of sequencing control and adult DM samples. A PCA was performed on CLR-transformed Aitchison distances. Distance between samples on the plot represents differences in microbial community composition, with 34.9% of total variance being explained by the first two components shown. DM samples are coloured by treatment groups, and sequencing control samples are red. The black ellipse represents the 95% confidence interval of the DM samples. Sequence variants are depicted by the gray numbers.
A

B

C

Shannon's Index

Aitchison distance within groups

Control
Control + BS168
1% Ox
1% Ox + BS168

Control
Control + BS168
1% Ox
1% Ox + BS168
Figure 43. Exploratory analysis of DM microbiota

A) Principal Component Analysis (PCA) plot of adult DM exposed to lithogenic media and BS168 supplementation. A PCA was performed on CLR-transformed Aitchison distances. Distance between samples (coloured points) on the plot represents differences in microbial community composition, with 36.3% of total variance being explained by the first two components shown. Strength and association for genera (sequence variants) are depicted by the length and direction of the gray arrows, respectively. Individual samples are coloured by treatment groups, and ellipses represent the 95% confidence interval of the diet groups. NF = Control diet, OX = 1.0% oxalate diet. B) Shannon’s Index of alpha diversity was calculated for each individual sample and plotted by treatment group. C) Intra-group Aitchison distance was determined within treatment groups. The distance of every individual sample to all others within the same treatment group was averaged to obtain a single distance value per sample. (B-C) Box plots illustrate the median, quartiles, and 5-95% confidence intervals. **P < 0.01, ****P <0.0001).
5.4.5 qPCR-based assessment of DM microbiota

No differences were determined between fly sexes for any taxonomic comparisons (data not shown), so pooled sex data were illustrated in Figure 44. Total bacterial and Wolbachia spp. loads were unchanged between treatment groups (Figure 44A and 44B, respectively). Abundance of the genus Lactobacillus was not significantly changed, however the genus Acetobacter was significantly decreased in the lithogenic diet groups (Figure 44C and 44D, respectively). Intra-individual ratio of Lactobacillus to Acetobacter was increased by the lithogenic diet ($P = 0.0007$); however, this phenomenon was rescued with BS168 supplementation (1% Ox- BS168 was not significantly different from untreated controls; $P = 0.048$ between 1% Ox- NA and 1% Ox- BS168) (Figure 44E). Species-specific primers were utilized to assess the load of Bacillus subtilis; BS168-treated groups trended towards increased loads, but these findings were not statistically significant (Figure 44F).
Figure 44. qPCR-based assessment of DM microbiota

Molecular quantification of total bacteria, bacterial genera, and species in whole-body DM adults (A-F). Intra-individual ratios of *Lactobacillus* : *Acetobacter* loads were compared in (E). All comparisons were made after normalizing to total host DNA. Data are depicted as mean ± standard deviation. Significance was determined with Tukey’s multiple comparisons tests. Each point represents a single adult DM, each from a separate experimental vial (n = 10, three technical replicates were performed). Sexes are pooled. * $P < 0.05$, *** $P < 0.001$. 
5.4.6 Effect of uropathogenic bacteria on stone burden in DM

Stone burden and health were assayed in the DM model of urolithiasis after supplementation with NaOx, UTI89, and PM175; adult survival, adult Malpighian tubule crystal birefringence, and larval locomotion were all evaluated (Figure 45). Kaplan-Meier survival analysis of adult DM (Figure 45A) demonstrated that UTI89 did not impact DM survival, however PM175 decreased DM survival irrespective of dietary lithogenesis. Larval crawling was not impacted by treatment with either uropathogen (Figure 45B). On day 7, dissected adult Malpighian tubules from UTI89-treated DM had significantly increased crystal deposition compared to untreated and PM175-treated DM (Kruskal-Wallis test with Dunn’s multiple comparisons, $P < 0.0001$ and $P = 0.001$, respectively) (Figure 45C-D).
A

Percent survival

Days

Control
Control + UTI89
Control + PM175
0.1% Ox
0.1% Ox + UTI89
0.1% Ox + PM175

B

Time to reach 3cm (s)

Control
Control + UTI89
Control + PM175
1.0% Ox
1.0% Ox + UTI89
1.0% Ox + PM175

C

Birefringent pixels/MT

0.1% Ox
0.1% Ox + UTI89
0.1% Ox + PM175

D

i) 0.1% Ox

ii) 0.1% Ox + UTI89

iii) 0.1% Ox + PM175

500 μm
**Figure 45. The effect of uropathogens on stone burden in DM**

A) Kaplan Meier survival analysis of five-day old DM that were supplemented with 2 X $10^8$ CFU of UTI89 or PM175 on Day 0, then transferred to normal or lithogenic media on Day 1 and followed until expiration. $n \geq 80$ per group from 3 biological replicates of $n = 20$-$30$ flies. B) Larval locomotion was determined on Day 4 after larvae were supplemented with 0.5 X $10^8$ CFU of UTI89 or PM175 on Day 0 and transferred to normal or lithogenic media on Day 2, $n = 20$ larvae per group from 3 biological replicates. C) CaOx crystals from polarized light microscopy of dissected Malpighian tubules were quantitated with ImageJ particle analysis, $n = 20$-$25$ adults per group from 3 biological replicates. D) Representative polarized light images of dissected Malpighian tubules from adult DM on Day 7 treated with 0.1% NaOx (i), 0.1% NaOx + UTI89 (ii) or 0.1% NaOx + PM175. The scale bar in D-iii) is applicable to all of D). All box plots illustrate the median, quartiles, minimum and maximum. *** $P < 0.001$, **** $P <0.0001$. 
5.4.7 Impact of microbes on renal calcium oxalate crystal adhesion and aggregation

MDCK renal epithelial cells were utilized to assess the effect of BS168, UTI89, and PM175 on adhesion and aggregation of calcium oxalate monohydrate (COM) crystals (Figure 46A). Cells that were pre-treated with BS168 prior to treatment with COM crystals in artificial urine did not show an increase in crystal adhesion compared to cells that were pre-treated with a media control (Figure 46B). The average crystal size was not significantly different between BS168 and PM175 treated groups and the untreated control, indicating that BS168 did not encourage aggregation (Figure 46C). In contrast, cells treated with UTI89 had significantly increased crystal adhesion and aggregation (Kruskall-Wallis test with Dunn’s multiple comparisons, $P = 0.016$ and 0.021, respectively) (Figure 46B-C).
A) Crystal aggregates were visualized with light microscopy after MDCK monolayers were pre-treated with MEM ± bacteria, followed by artificial urine ± 5 mg/mL COM (Ox). Scale bars are 200 µm. B) Amount of adhered COM crystals to MDCK cells was not significantly different between Ox-BS168 or Ox-PM175 treated groups compared to Ox alone but was significantly higher for Ox-UTI89 (Kruskall-Wallis test with Dunn’s multiple comparisons). C) Average crystal aggregate size was not significantly different between Ox-BS168 or Ox-PM175 treated groups compared to Ox alone but was significantly higher for Ox-UTI89 (Kruskall-Wallis test with Dunn’s multiple comparisons). Three technical and three biological replicates were performed (n = 9). Box plots illustrate the median, quartiles, minimum and maximum. *P < 0.05.

Figure 46. Effect of microbes on COM crystal adhesion to renal epithelial cells
5.4.8 Development of a live imaging µCT scanning protocol of DM

The fruit flies tolerated three but not six or eight hours of constant flow of CO₂ anesthesia in combination with X-ray radiation exposure throughout the µCT acquisition. During this time, the optimal scanning protocol achieved DM survival and minimal image-noise (Figure 47A). With this method, successful 3D reconstruction and visualization of DM adults were reproduced at 5.72 µm isotropic voxel spacing with stones clearly present; thus, this protocol was utilized for experimental scans.

Adult DM were exposed to 1.0% Ox lithogenic media and BS168 supplementation as described above. Four adult males from each group were then selected for repeated µCT to validate the live, repeated scanning methodology (Figure 47C). After scanning it was apparent that the µCT detected calcification in the DM legs, head, and thorax, which was photoshopped out of the images prior to analysis, such that stone quantification only involved calcification within the abdomen. The calculated stone volume was unchanged in the 1% Ox group between scans, but surprisingly was decreased on day 7 compared to day 3 in the 1% Ox + BS168 group ($P = 0.48$ by unpaired, two tailed t-test) (Figure 47B).
Figure 47. Repeated live imaging by μCT demonstrates stone burden in DM

A) DM survived less than six hours of CO₂ anesthesia (n = 8 for each timed scan). Images were acquired under constant flow of CO₂ (0.5 mL/min), over 360° (0.4° incremental angle). Survival was counted immediately after the scan and DM were followed for 3 days. On day 6 and 10, DM that had undergone a 3h scan had 100% survival. B) Quantification of stone burden in DM. Stone volume was calculated as the sum of all pixels >700 HU within the 3D CT reconstructed images. Stone size was unchanged between scans for OX-NA cohort but decreased from Day 3 to Day 7 scans in the OX-BS168 cohort by unpaired, two tailed t-test. n = 4 DM per cohort, one experimental replicate performed. C) Representative 3D CT reconstruction of live anesthetized male DM demonstrating the feasibility of repeated scanning of the same individual flies longitudinally. 3D volume representations were reconstructed at 5.7-micron isotropic voxel size. Colour code is Hounsfield Units (HU), with structures >700 HU coloured red. Calcification outside the abdomen (not stones) are visible in all groups. Box plots illustrate the median, quartiles, minimum and maximum. *P < 0.05.
5.5 Discussion

This study is the first to characterize the role of both beneficial and pathogenic microbes in a *Drosophila melanogaster* (DM) model of urolithiasis, in a step towards understanding the host-microbe interactions involved in kidney stone disease. The *Bacillus subtilis* strain 168 (BS168) ameliorated stone burden in DM. Notably, growth of BS168 increased in the presence of sodium oxalate, and BS168 increased adult DM survival, increased markers of health in DM larvae, decreased stone burden in DM Malpighian tubules and fecal excreta, as well as altered the adult DM microbiota. In contrast, a clinical kidney stone isolate of *Proteus mirabilis* (PM175) decreased DM survival irrespective of lithogenesis, and the uropathogenic *Escherichia coli* (UTI89) increased stone burden in DM and aggravated crystal deposition to renal epithelial cells *in vitro*. A novel, sensitive, live imaging modality was developed for DM using μCT. Collectively, the *in vivo*, *in vitro*, and imaging findings suggested that BS168 may mitigate and UTI89 may contribute to, urolithiasis development and severity. This further supports a potential role for BS168 as a future therapeutic adjunct in the treatment of human nephrolithiasis.

Oxalate is a toxin consumed in the diet and produced endogenously in the liver (Hatch and Freel, 2008). It can act as a chelating agent of metallic cations and can impart toxic effects by means of altered gene expression, membrane disruption, and production of reactive oxygen species, among other methods (Hess *et al*., 1998; Jonassen *et al*., 2005; Miller and Dearing, 2013). Humans are incapable of degrading oxalate and instead rely on excretion and microbial degradation (Miller and Dearing, 2013); accordingly, it is a common component in kidney stones (Moe, 2006). Many species of bacteria are able to degrade the compound; however, oxalate can still exert toxicity over bacterial cells and many gut commensals cannot tolerate high levels of oxalate (Suryavanshi *et al*., 2016). Here, it was demonstrated that BS168 exhibited prolific growth in media with up to 50 mM NaOx present, which is expected to far exceed physiological relevance based on an approximated 2.0 mM daily dietary oxalate ingestion (Holmes and Assimos, 2004). Alongside its ability to form endospores, resist bile salts, and tolerate low pH conditions, these results suggest that BS168 would likely survive well in the human intestinal tract.
despite even the highest levels of dietary oxalate consumption (Jeon et al., 2017). Hatch and colleagues (2011) demonstrated that the gut commensal bacterium *Oxalobacter formigenes* not only degrades oxalate, but also promotes enteric oxalate excretion from circulation. Future work on BS168 should investigate this potential and evaluate the degree to which BS168 may lower serum and urinary oxalate concentrations.

In the well-established DM model of urolithiasis, BS168 was able to transiently colonize the intestinal tract for up to 5 days following a single treatment yet it could elicit marked improvements to DM survival during at least 14 days of lethal oxalate exposure (Ali et al., 2018; Chen et al., 2011; Miller et al., 2013). These findings suggest that, in addition to directly metabolizing oxalate, BS168 may reduce oxalate toxicity indirectly through priming of host cell physiology (Tanner and Bornemann, 2000). It is well known that renal oxalate toxicity is primarily mediated via mitochondrial dysfunction and excessive reactive oxygen species (ROS) generation propagated by phospholipase A2 (PLA2) activation (Cao et al., 2004). This process leads to inflammation and damage to the renal epithelium, which can become a crystal deposition site, accelerating stone formation (Albert et al., 2020; Miller et al., 2000; Zuo et al., 2011). Notably, *B. subtilis* can biosynthesize lipopeptides that are potent inhibitors of PLA2, which have been demonstrated *in vivo* to decrease inflammation (Selvam et al., 2009; Volpon et al., 2000). This suggests that the survival benefits afforded by BS168 in oxalate-exposed DM may be partly due to the prevention of oxalate-induced mitochondrial dysfunction via blunting of the PLA2-facilitated ROS signaling cascade.

In corroboration with these findings, BS168 increased psychomotor activity, indicative of improved metabolic energy conversion and neuronal development, in both oxalate-exposed and non-exposed DM larvae (Clark et al., 2018; Jakubowski et al., 2012; Yang and Hultmark, 2017). Given the integral role of PLA2 in modulating oxidative stress-related degenerative diseases in DM, these results indicate that BS168-mediated modulation of PLA2 activity may represent a key mechanism of indirect protection against oxalate-based stone disease (Iliadi et al., 2018). Utilizing alternative mechanisms, recent evidence has also shown that *B. subtilis* from fermented foods can decrease mitochondrial dysfunction, oxidative stress, and DNA damage associated with metabolic
dysfunction (Prazdnova et al., 2015; Do et al., 2015; Boyd et al., 2018). Given the links between metabolic syndrome and urolithiasis, the conserved and multipronged ability of *B. subtilis* strains to promote mitochondrial health may offer a simple and effective solution for attenuating oxalate-induced renal damage in patients with recurrent stone formation (Boyd et al., 2018; Ramaswamy and Shah, 2014).

Oxalate has been shown to alter the microbiota in humans, however, how this influences the microbiota in nephrolithiasis is still unclear (Miller and Dearing, 2013; Suryavanshi et al., 2016). For this reason, the simplicity of the DM urolithiasis model was advantageous for evaluating the effect of the lithogenic diet on the microbiota. Indeed, it was found that oxalate consumption could exact significant effects to the DM microbiota, some of which were mitigated by supplementation with BS168. DM exposed to the lithogenic diet yielded significantly lower culturable bacterial CFU; this was in contrast to unchanged total bacterial loads that were determined by qPCR. However, the flies were known to be heavily colonized by intracellular insect commensal *Wolbachia* spp. which accounts for this discrepancy, as this genus accounts for the majority of the qPCR load but is not cultivable in standard bacterial culture, instead requiring propagation in insect cell lines (O’Neill et al., 1997). Consumption of the lithogenic diet by adult DM also led to significant alterations in the microbial alpha and beta diversity, and additionally altered the ratio of the two dominant bacterial genera of the DM microbiota, *Lactobacillus* and *Acetobacter*. In both instances, BS168 was able to rescue these phenotypes. These findings suggest that BS168 exerts its protective effects without saturating the DM microbiota. Supporting this, BS168 was undetectable by culture from DM beyond experimental Day 5, while on Day 7, no sequence variants corresponding to *Bacillus* spp. were detected with 16S rRNA gene sequencing, and by qPCR, the *B. subtilis* loads were nearing the limits of detection and comparable between all groups. This is an important feature of *B. subtilis*, because as an oxalate-tolerant spore former, the potential existed for it to overtake the endogenous microbiota in a manner reminiscent to *Clostridium difficile* infection in humans, however this was not observed (Voth and Ballard, 2005).
For decades, struvite stones have been referred to as “infectious” stones, due to their association with urinary tract infections (UTIs) (Thompson and Stamey, 1973). Organisms such as *Proteus mirabilis*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Staphylococcus saprophyticus*, and *Ureaplasma urealyticum* produce urease, an enzyme that cleaves urea to ammonia and CO$_2$, which subsequently elevates urinary pH and precipitation of magnesium ammonium phosphate hexahydrate crystals (Das *et al*., 2017; Thompson and Stamey, 1973). Until more recently, bacteria were not thought to contribute to the formation of non-struvite stone compositions (Coe *et al*., 1992; Schwaderer and Wolfe, 2017). Groups have now validated the presence of live bacteria within stones of various compositions with both culture and molecular techniques, and we have corroborated these findings (Chapter 3) (Barr-Beare *et al*., 2015; Dornbier *et al*., 2019; Golechha and Solanki, 2001; Sohshang *et al*., 2000; Tavichakorntrakool *et al*., 2012; Wang *et al*., 2014; Zampini *et al*., 2019). Based on results from our group and others, *E. coli* appears to be the most commonly isolated microbe from non-struvite stones, followed by *Pseudomonas* and *Staphylococcus* spp. Whether these microbes play an intimate role in stone formation, or if they are just passive bystanders, has not been well described. The concept of such well-known pathogens being bystanders is hard to imagine. Potentially, while not being metabolically active enough to induce a classical infection, they could still be causing an element of harm to the host. For example, very low abundances of *Pseudomonas*, *E. coli* and other pathogens have been identified in breast tissue of women with cancer despite no infectious process taking place (Urbaniak *et al*., 2014).

The DM urolithiasis model proved useful in revealing that PM175 had a detrimental effect on DM survival, irrespective of lithogenesis as both PM175 alone and 0.1% Ox + PM175 groups had similar mortality, and 0.1% Ox + PM175 did not have increased stone formation compared to 0.1% Ox alone. This was unexpected, as *P. mirabilis* is not a known pathogen of DM (although its close relatives *Providencia* spp. are) and it was only supplemented at the initial Day 0 timepoint (Galac and Lazzaro, 2012; Ma *et al*., 2012). It is possible that this microbe exerts a pathogenic role on DM during a key developmental window in young adult flies, which could result in subsequent mortality effects later in life regardless of stone formation (Clark *et al*., 2015). In contrast to the findings in
PM175, we found UTI89 to significantly increase stone deposition in a manner that did not affect DM survival. This may indicate that the DM urolithiasis model is already quite severe, and although UTI89 impacts stone formation, it may not manifest worse mortality beyond that of the lithogenic diet alone (Miller et al., 2013).

Previous studies with uropathogenic *E. coli* (UPEC) have demonstrated that they aggregate on and around CaOx monohydrate (COM) crystals, significantly more so than other crystal compositions (Barr-Beare et al., 2015; Chutipongtanate et al., 2013). Other groups have shown the ability for COM to adhere to MDCK renal epithelial cells, but the role of bacteria in this process had not been previously explored (Bigelow et al., 1998; Yamaguchi et al., 2002).

It is unlikely that a large quantity of BS168 would be present in the kidney after oral administration, even though the gut is a reservoir for the urogenital microbiota (Yamamoto et al., 1997). However, as gut colonization with UPEC has been shown to increase the risk of UPEC UTI, experiments were performed here to address how BS168 may impact the urinary tract and stone development should some cells traffic there (Moreno et al., 2006; Moreno et al., 2008). Here, BS168 did not encourage aggregation nor adherence of COM crystals to the MDCK cells. Accordingly, these *in vitro* findings suggest that if any orally consumed BS168 cells did migrate to the urinary tract, increased morbidity would be unlikely.

Conversely, in agreement with previous UPEC work by Barr-Beare et al. and Chutipongtanate et al., UTI89 demonstrated the ability to aggregate COM crystals and encourage their adherence to renal epithelial cells (2015; 2013). This demonstrates how UPEC, unlike other uropathogens such as PM175 which had no appreciable effect on the COM crystals, could be actively involved in CaOx stone growth through its ability to potentiate crystal deposition. This may be the consequence of active bacterial adhesion to the crystals, charge interactions between the bacterial cell surface and urinary ions, or the expression of enzymes such as citrate lyase, which through its ability to convert citrate to acetyl-CoA can increase urinary CaOx supersaturation (Bayer and Sloyer, 1990; Chutipongtanate et al., 2013; Quentmeier et al., 1987; Zuckerman and Assimos, 2009).
Future research should investigate the microbes present in non-struvite kidney stones in the context of urine chemistry to determine if hypocitraturia is a significant, uropathogen-induced factor leading to stone formation or recurrence (De Ferrari et al., 1996).

Finally, this study was the first to demonstrate the ability to live image DM using a novel anesthetic and µCT protocol. Just as CT scans are used to visualize stones in human, previous studies have utilized X-ray µCT to evaluate stones in narcotized, wax-embedded DM (Assimos et al., 2016; Hirata et al., 2012). Unfortunately, anatomical structures are often difficult to appreciate in narcotized flies and this method precludes longitudinal studies. Here, the successful application of an acrylic scanning apparatus enabled anesthesia and full-body immobilization of up to 16 flies at a time, with 100% recovery after two 3-hours scans. This methodology enabled repeated scanning and the capability of time course studies in DM. Although as an overall urolithiasis model DM are exceedingly economical, the use of flies as a high-throughput screening tool for the assessment of expensive pharmaceutical or nutraceutical stone therapies can still amount to significant costs (Miller et al., 2013). The ability to repeatedly scan DM and follow stone progression longitudinally throughout life is a significant advancement to the field.

In summary, this study has characterized the beneficial properties of BS168, and the uropathogenic stone-promoting effects of UTI89 in the context of nephrolithiasis, as assayed in a DM model of the disease and in vitro cell culture experiments. This validates that in the capacity of both probiotics and pathogens, microbes can play an instrumental role in stone prevention and formation, respectively. Although probiotics are classically Lacotobacillus spp. or Bifidobacterium spp., strains of Bacillus subtilis are generally regarded as safe, gaining favour as probiotics for gut-related maladies, and are components of several fermented foods (Kim et al., 2014; Poormontaseri et al., 2017; Sorokulova et al., 2008). To date, studies employing various formulations of probiotics in nephrolithiasis patients have largely been inconclusive due to the broad variety of preparations tested alongside ill-defined strain selection, thereby making it unclear how efficacious this approach could be (Abratt and Reid, 2010; Campieri et al., 2001; Duncan et al., 2002; Jairath et al., 2015; Lieske et al., 2010; Okombo and Liebman, 2010). Instead, future studies should carefully evaluate mechanistically validated strains which
can be delivered effectively to the gut, such as BS168. Based on our seminal findings, this microbe may prove a novel therapeutic adjunct to reducing the incidence of recurrent CaOx nephrolithiasis in high-risk patients.

5.6 References


Chapter 6

6 General Discussion

The study of the human microbiome is regarded as one of the most pivotal scientific developments of the 2010’s (Nature, 2019). The advances in human microbial ecology have been aided by the information revolution, the advent of next generation sequencing (NGS) technologies, and big data analysis (Malla et al., 2018). Thanks to these innovations, we now know that the microbial “organ” living within us (the microbiota), and its genes and products (the microbiome), are intimately involved in human health and disease (Malla et al., 2018; Whiteside et al., 2015). In this thesis I provide evidence that the human microbiota at multiple anatomical sites is implicated in kidney stone formation and can be exploited in this disease’s treatment and prevention.

6.1 Towards standards in microbiota studies

Along with the rapidly developing discipline of microbiota research has come a wide variety of study methodologies, none of which are standardized across the field (Goodrich et al., 2014; Hiergeist et al., 2016). These differences in protocols extend from study inception and span through to the final publication, with areas of potential bias at every step along the way. These can come from sample collection (further diversified by biological sample type), sample processing and storage, DNA extraction, PCR amplification, sequencing, and bioinformatic analysis. Coupled with the sensitivity of NGS, these factors can make cross-comparison between studies very difficult, and meta-analyses nearly impossible. In this thesis, urinary, gut, medical device, and in vivo model samples were investigated, all with the aim of making the highest fidelity 16S rRNA and whole shotgun metagenomic sequencing feasible. With these factors in mind, careful consideration was taken at all levels of study design and performance to yield next generation sequencing analysis to the very best of our ability.

Previous studies in our lab by Dr. Yige Bao worked towards optimizing the handling of urine samples, as well as similar clinical samples with high host- and low microbial-biomass (Bao, 2018; Minich et al., 2019; Wolfe et al., 2012). These findings suggested
that voided urine retained optimal microbial resolution for studies with 16S rRNA gene sequencing when samples were greater than or equal to ten millilitres in volume and were centrifuged, with the supernatant discarded, retaining a pellet for frozen storage at -80 °C. Employing this method resulted in consistent, high quality sequencing results even after samples spent two years in frozen storage (Bao, 2018). Therefore, this method was utilized for the urine sample collection in Chapters 3 and 4 of this thesis.

Although the bacterial biomass of feces is high, unlike most urinary samples, these samples present a different set of challenges (Sender et al., 2016). Study subjects are usually willing to provide urine samples, but less so fecal samples “on demand” per se, so at-home collection is commonly required. This was the case in the kidney stone study (Chapter 3), where fecal samples were to be collected prior to commencing pre-surgical antibiotic prophylaxis, as these drugs would affect the microbiota composition with consequences for the subsequent analysis. At-home collection is usually more amenable to participants than in-clinic deep rectal swabs, although both methods appear to approximate the gut microbiota comparably (Biehl et al., 2019; Liang et al., 2020). Whether participants collect a full fecal sample, just a portion on toilet paper, or a swab thereof is a second point of review, although again method acceptance by the participant should be considered to optimize study recruitment (especially where monetary incentives aren’t offered, as in the clinical studies performed in Chapters 2, 3, and 4) (Gilbert et al., 2014; Liang et al., 2020). Further confounders include interim storage of these samples and whether same-day lab delivery, or perhaps mailing of a sample is sufficient (Gilbert et al., 2014; Liang et al., 2020; Voigt et al., 2015). For this reason, Chapter 2 evaluated the feasibility of soiled toilet paper collection and storage for the purpose of NGS. Fecal samples were homogenized and transferred evenly to toilet paper, then stored dry or in the preservative agent RINAlater® at various temperatures and for various lengths of time. This study validated that unpreserved fecal samples collected on soiled toilet paper and stored in sterile sample bags are appropriate for NGS after being mailed. Specifically, it was determined that the length of time and temperatures experienced by unpreserved samples mailed via Canada Post would still resemble the origin with minimal variation. This methodology has since been utilized in numerous clinical studies from our group, including those of the gut microbiota in irritable bowel
syndrome, multiple sclerosis, non-alcoholic fatty liver disease, schizophrenia, chronic pain, and of course, nephrolithiasis (Craven et al., 2020).

6.2 The microbiota of recurrent nephrolithiasis patients diverges from a healthy state

A primary purpose of this thesis was to characterize how the microbiota is altered in kidney stone patients in order to elucidate whether bacteria are implicated in stone formation. Utilizing the methods established in earlier work, Chapter 3 involved a microbiota-targeted clinical study of both healthy adults and kidney stone patients receiving surgical treatment. Despite the publication of several studies aimed at addressing similar or associated questions since the onset of this thesis, a certain consensus has not been reached by others (Barr-Beare et al., 2015; Batagello et al., 2018; Dornbier et al., 2019; Magwira et al., 2012; Miller et al., 2019; Stern et al., 2016; Suryavanshi et al., 2018; Tang et al., 2018; Tavichakorntrakool et al., 2012; Ticinesi et al., 2018; Xie et al., 2020; Zampini et al., 2019). These studies have few similarities and many discrepancies between their design and results, epitomizing how the lack of standards in the microbiome research field can lead to faulty conclusions. To avoid such issues, this thesis sought to combine the individual strengths of several of these studies (large sample size, sampling at multiple timepoints and anatomic sites, dietary consideration, the combination of both 16S rRNA gene and whole shotgun metagenomic sequencing, and scrupulous bioinformatic analysis) into a single investigation that should stand up against re-examination with reproducibility in future studies.

In this study, stone patients were found to have higher urinary oxalate concentrations compared to healthy subjects, despite similar dietary micronutrients consumption by both cohorts. In an effort to understand this finding, further investigation showed that the urinary and gut microbiota were distinct in kidney stone formers compared to healthy subjects. Based on the differential features, the microbiota is believed to play a significant role in nephrolithiasis. During the time period of surgical stone management, the urinary microbiota was enriched by bacterial genera known to be associated with inflammatory responses. In addition, a sequence-positive microbiota was detected within all crystalline compositions of kidney stones examined. Based on literature expectations,
it was surprising to not detect a lower relative abundance of *Oxalobacter formigenes* in the gut microbiota of stone formers. This further questions the correlation between this species and prevention of stone formation.

A novel finding was the higher relative abundance of antibiotic resistance factors and uropathogenic bacteria, as well as previously unreported functional changes involved in oxalate homeostasis indicative of metabolic stone formation. Together, these findings implicate the gut as a reservoir for the urinary tract and stone formation. Interestingly, surgical stone management, which invariably includes antibiotic use, significantly disrupted the urinary and gut microbiota and led to blooming of antibiotic resistant uropathogens in the urinary tract, facilitating further crystal nidi development and disease recurrence, a finding observed epidemiologically (Johnson *et al.*, 1979; Koşar *et al.*, 1999; Vaughan *et al.*, 2019).

**6.3 The microbiota and kidney stone disease management**

A key step in applying microbiome findings to the clinic is to understand how the microbiota might influence kidney stone disease management. An almost ubiquitous component of such care involves ureteral stent placement. These stents along with urinary catheters are the most commonly used medical devices in urological practice and the most common source of device and hospital-acquired infections (Chatterjee *et al.*, 2014; Lo *et al.*, 2014). In spite of the risk of infection, encrustation, and the morbidity associated with ureteral stent placement, they are used for nearly all nephrolithiasis patients at some point throughout their treatment, most routinely following surgical stone intervention (Assimos *et al.*, 2016; Dyer *et al.*, 2002; Halebian *et al.*, 2008). Thus, in Chapter 4, the goal was to characterize the microbiota adhering to ureteral stents and determine which, if any, patient factors influenced the microbiota composition and stent encrustation.

Upon attempting to evaluate all ureteral stents removed from our centre over a one-year period, ultimately 241 patients were enrolled, of which 91% were receiving treatment for urolithiasis. Both mid-stream urine and ureteral stents were collected and characterized
with 16S rRNA gene sequencing. The presence of a reproducible, patient-specific stent microbiota was validated, even in visibly un-encrusted devices. Device indwelling time, patient age and comorbidities such as metabolic syndrome, irritable bowel syndrome, inflammatory bowel disease, and pulmonary disease were all significantly associated with stent and urinary microbial community variation. Unexpectedly, the use of antibiotics by participants throughout the course of stent placement did not alter the microbial community composition, microbial diversity, or degree of stent encrustation. Because antibiotics are often prescribed alongside stent placement and removal in an attempt to curb urinary tract infections (UTIs) and device encrustation, these findings raise questions about the necessity of this standard practice (Abbott et al., 2016; Riedl et al., 1999).

6.4 The ramifications of disrupting the microbiome
The finding that recurrent kidney stone formers have altered bacterial community composition in both the intestinal and urinary tracts markedly correlates with recent findings that oral antibiotics increase the risk of nephrolithiasis (Tasian et al., 2018). Moreover, findings in stent patients revealed that antibiotic use did not create a health-associated urinary microbiota devoid of uropathogens, nor did it decrease device encrustation. Instead, the overall results raised potential disadvantages and long-term consequences of antibiotic use in urological practice. This is not to suggest that antibiotics do not fulfill a necessary and invaluable role in disease treatment (López Romo and Quirós, 2019; Paterson et al., 2016). However, the use of these agents remains debated for many urological scenarios — a particularly consequential fact given that urologists have higher antibiotic prescription rates than providers from any other surgical specialty (Durkin et al., 2017). Additionally, antimicrobial use by urologists is often discordant to the best practice guidelines published by the American Urological Association (AUA) (Khaw et al., 2018; Wolf et al., 2008). Clearly, the reasons for this must be examined given the knowledge emerging from microbiome studies, including those described here.

Urinary tract infection is the second most common indication for all antibiotic prescriptions behind respiratory infections (Shively et al., 2018). In studies of antibiotic
use for acute uncomplicated UTIs, randomized placebo-controlled trials have demonstrated that patients given placebo exhibit significant symptom improvement and cure of bacteriuria after just days. Thus, uncomplicated UTIs are often cleared without drug treatment (Christiaens et al., 2002; Ferry et al., 2004; Little et al., 2010). Furthermore, antibiotic use does not provide significant benefit for UTI prevention in ureteral stent patients with normal risk profiles, yet they are still commonly prescribed for this indication (Abbott et al., 2016; Khaw et al., 2018; Lee et al., 2019). Similar controversy surrounds antibiotic use in ureteroscopic lithotripsy, transurethral resection of the prostate, and even PCNL (Baten et al., 2019; Deng et al., 2017; Khaw et al., 2018; Potretzke et al., 2016). Further, none of these studies have considered the damage incurred by antibiotics to the microbiome and to the patient’s health as a consequence of this.

It is known that the use of antimicrobials can cause pronounced and long-lasting microbiota remodelling, which can have implications for host health for years to come (Cho et al., 2012; Cox et al., 2014; Dethlefsen and Relman, 2011; Korpela et al., 2016; Ruiz et al., 2017). Although much literature has focussed on oral antibiotic prescription, this is not where the assault to our microbiome ends (Lozupone et al., 2012). In Western society, people are constantly bombarded by antimicrobial substances, from prescription and over-the-counter medications, to preservatives in food, and even contaminants in drinking water (Khan and Nicell, 2015; Lozupone et al., 2012; Maier et al., 2018; Nash, 2012). Many negative consequences of microbiome-disrupting substances have been reported to affect host metabolic syndrome, psoriasis, asthma, allergies, and intestinal diseases, among many others (Brown et al., 2013; Cho et al., 2012; Cox et al., 2014; Ni et al., 2019; Schulfer et al., 2018; Zanvit et al., 2015). Now, we also know there to be a direct role in urological conditions (Tasian et al., 2018). Even when used appropriately, antibiotics can lead to subsequent infection by resistant organisms in the patient, as well as the dissemination of resistant organisms in the community — a separate but very significant global public health issue (Barancheshme and Munir, 2018; Costelloe et al., 2010). Together, these factors lead to a multi-pronged depletion in the diverse beneficial microbiome, reduction of key functional pathways, and enrichment of inflammatory, drug-resistant pathogens (Lozupone et al., 2012).
Simply put, substances capable of disrupting the microbiome are likely capable of causing disease. The net effect of disruption has been termed “dysbiosis”, but this is poorly defined and relatively uninformative, as there is no single healthy microbiome nor an all-encompassing harmful one. The relationships between the microbiome and diseased states are often not defined mechanistically. This knowledge gap with regards to a mechanistic role of uropathogens and stone formation was investigated in Chapter 5 using a *Drosophila melanogaster* (DM) model of stone formation and *in vitro* tissue culture experiments. It was discovered that the *Escherichia coli* strain UTI89 and a struvite kidney stone isolate of *Proteus mirabilis* (PM175) had very different outcomes. The DM stone burden along with calcium oxalate monohydrate (COM) crystal aggregation and adhesion to renal epithelial cells increased significantly with UTI89. Whereas, PM175 increased DM mortality but did not exacerbate stone formation, and trended towards increased COM crystal adhesion, though that result was not statistically significant. Although only two uropathogenic bacteria were tested, these results demonstrated that the strains had vastly different mechanisms of action in their ability to potentiate kidney stone formation: one by a urease-mediated increase in urine pH (PM175), and the other potentially by bacterial adhesive mechanisms or citrate lyase-mediated hypocitraturia (UTI89). It is mechanistic studies such as these that will push the microbiome research field beyond simply exploratory characterization, and towards hypothesis driven investigations and the development of biologically relevant solutions.

### 6.5 Mitigating microbiome damage

Although the negative impact of our ‘Western’ lifestyle on the microbiome may seem overwhelming, all hope is not lost. It is through first understanding the microbial involvement in human diseases and defining the relevant mechanisms of action, that we can then work towards targeted microbial-based therapeutics. Probiotics, or “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host”, are just one potential course of action (Hill *et al*., 2014; WHO, 2001). It is important when investigating potential probiotic bacteria that they are evaluated for strain-specific niche applications. Where past studies have failed to demonstrate the benefit of a potentially beneficial microbe, they have often lacked a rationale for strain
selection (Suez et al., 2018). Indeed, although probiotics likely would not offer a community-wide shift from a depleted microbiota, they could be best utilized for replenishment of a specific functional outcome, such as repair a loss-of-function phenotype of oxalate degradation in stone-formers.

In Chapter 5 of this thesis, *Bacillus subtilis* strain 168 (BS168), a resilient spore-forming bacterium with the capability to degrade oxalate through the enzyme oxalate decarboxylase, was investigated (Sorokulova et al., 2008; Tanner and Bornemann, 2000). The rationale was that oral consumption of this microbe could decrease urinary oxalate concentrations by degrading dietary oxalate in the gut. *O. formigenes* and other oxalate-degrading bacteria are currently sold as “probiotics” for this purpose, although trials and validation studies have had underwhelming results, potentially due to *O. formigenes*’ strict anaerobic nature and difficulty in retaining its viability (Ellis et al., 2015; Milliner et al., 2018). Unlike current products on the market, BS168 was able to grow in very high levels of oxalate, reduce stone burden, and improve markers of health in our DM model of the disease (Ellis et al., 2015). This microbe is also a component of various fermented foods (Kim et al., 2014). Based on these findings and the fact that *Bacillus* spp. are gaining favour as probiotics for other purposes in humans, BS168 should be further evaluated for its therapeutic efficacy and safety in humans with nephrolithiasis (Poormontaseri et al., 2017; Sorokulova et al., 2008).

### 6.6 Future directions

Beyond probiotics, there are numerous other techniques available to reshape a damaged microbiome. These could include prebiotics, “a substrate that is selectively utilized by host microorganisms conferring a health benefit”, or whole community transformation via fecal microbiota transplant (FMT) (Gibson and Roberfroid, 1995; Gibson et al., 2017). With recent interest in the gut microbiota of nephrolithiasis patients has come curiosity surrounding the therapeutic potential of FMTs (Miller et al., 2017; Stern et al., 2019). As of yet, this has only been investigated in animal models, which tend to poorly recapitulate the complex human disease phenotype (Khan, 1997). As leaders in the field of FMT research (having established studies involving FMT for non-alcoholic fatty liver disease, multiple sclerosis, schizophrenia, and cancer immunotherapy, among others), as
well as utilizing the technique clinically for *C. difficile* infection, our centre has the potential to be the first to investigate this therapy in humans with stone disease (studies ongoing; Craven *et al.*, 2020).

A major point of attention when undertaking an FMT study is appropriate donor selection. Donors are routinely screened and excluded based on relevant diseases, family history, and detection of transmissible agents (Craven *et al.*, 2017; Duvallet *et al.*, 2019; Woodworth *et al.*, 2017). However, the broader recommendations of what an appropriate donor looks like based on the disease of focus is often unclear, when we know for example that such phenotypes as leanness and obesity could be transferred through the microbiome (Vrieze *et al.*, 2012). Without knowing what a healthy microbiome actually is, how can an optimal donor be identified? The criteria should encompass much more than simply a lean person that does not harbour *Helicobacter pylori* or HIV.

It is hoped that the research undertaken in this thesis will advance the field in several ways. For example, based on the findings herein, the ideal FMT donor for a middle aged, male, nephrolithiasis patient should likely be: middle aged, male, with no family history of stones or urological conditions, consuming a healthy diet, physically active, hasn’t used antibiotics in the last 5 years, has a robust gut microbiota oxalate degradation network (i.e. *Oxalobacter, Lactobacillus,* and *Bifidobacterium* spp., or *Bacillus subtilis*), has low to normal urinary oxalate concentration, absent or low numbers of uropathogenic bacteria in the gut, absent or low numbers of antibiotic resistant organisms in the gut, robust gut microbiota capacity for vitamin production (i.e. B6), with reduced ascorbate utilization. The search for donors who match such criteria based on the recipient may be exceptionally expensive and challenging, and weighing each element may require some mathematical modeling. But ultimately, a study could test FMT from such a disease-based selection process to determine whether stone recurrence rates in a patient population are decreased after receiving the autologous vs. an allogeneic transplant.

In addition to investigating FMT as a potential therapeutic, many of the other findings in this work warrant further investigation and *in vitro* validation. To confirm the gut microbiota findings and functional differences detected by shotgun metagenomic
sequencing, a dynamic host-free model system of the gut (a chemostat) should be enlisted (Allen-Vercoe, 2013). In this in vitro culture vessel system that mimics microbial metabolism in the distal colon, stool from both healthy controls and recurrent stone patients could be separately investigated and compared for their functional capacity. For example, the concentration of free oxalate could be quantitated after dosing the systems with a known quantity of ascorbate (the oxalate concentration could be measured in chemostat effluent using HPLC, as was done in Chapter 3). Similarly, the capacity of the respective microbiomes to generate relevant micronutrients could be assessed following the addition of a standard media “meal” (i.e. concentrations of Vitamins B₆ or K₂, which could be quantitated with ELISA).

Future studies should help to clarify the contribution of uropathogens to stone formation. In chapter 5, renal epithelial cell culture and COM crystal adhesion assays were combined to evaluate how bacteria may be directly interacting with crystals. These were quite short experiments by nature as the renal cell line and bacteria could not co-exist long term. A stone generator apparatus, as described previously, might prove to be a better way to investigate longer interactions (Ananth et al., 2002; Chow et al., 2004). This system involves the growth of crystal nidi into stones of clinically relevant size, in chambers supplied with COM-supersaturated artificial urine. Different urinary components, macromolecules, and stone types could be investigated simultaneously and multiplexed into a so-called “stone farm” (Ananth et al., 2002; Chow et al., 2004). Bacteria could be applied to the system at different stages of stone development, with absolute stone size and growth rate as a functional experimental output. Additionally, these experiments would benefit from image analysis, whereupon bacteria could be fluorescently labelled, and stones sectioned and imaged microscopically (Sivaguru et al., 2018).

What potentially makes the bacteria isolated from stones different from bacteria present in the bladder of healthy people is also of great interest. In Chapter 3, the genera *Escherichia, Gardnerella*, and *Staphylococcus* were sequenced and cultured from both healthy and stone forming participants’ urine samples, as well as the kidney stones themselves. These isolates could undergo whole genome sequencing to determine if
species or strain-specific traits are differentially pathogenic between the two cohorts. Perhaps the *E. coli* from stone patients harbour more adhesive pili, invoke inflammation or host tissue damage through toxin production, or have enhanced antimicrobial resistance compared to isolates from healthy participants (Wiles *et al*., 2008). Genomic differences could be investigated mechanistically using the *in vitro* models described above to determine their biological significance.

6.7 Concluding remarks

As we further our understanding about the intimate role that microbes play in human health, nephrolithiasis is an extremely important area to apply this knowledge. This often-idiopathic condition may be better understood in the context of microbes, with beneficial ones harnessed as part of nephrolithiasis treatment and prevention. This disease affects a tremendous amount of people and causes severe morbidity and economic strain; its prevalence is only increasing, and we need novel solutions. This thesis provides information upon which to not only base future research, but also to attract the attention of urologists at the front line. It is my sincere hope that the work presented in this thesis (as well as the other publications resulting from my graduate studies) have advanced understanding of the microbiota, the 80th human organ, and its intertwined relationship to human health (Standring *et al*., 2015).

6.8 References


course has lasting effects on murine microbial network topology and immunity. Nat. Commun. 8, 518.


Appendix A: Content license from Journal of Microbiological Methods

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Appendix B: Ethical approval for kidney stone clinical study

Western Research
Western University Health Science Research Ethics Board
HSREB Delegated Initial Approval Notice

Principal Investigator: Dr. Hassan Razvi
Department & Institution: Schulich School of Medicine and Dentistry/Surgery, Western University

HSREB File Number: 105443
Study Title: Microbiome and Metabolome Biomarkers of Kidney Stone Formation
Sponsor: St. Joseph's Healthcare Foundation

HSREB Initial Approval Date: December 23, 2014
HSREB Expiry Date: December 23, 2015

Documents Approved and/or Received for Information:

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The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above named study, as of the HSREB Initial Approval Dateneat above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review. If an Updated Approval Notice is required prior to the HSREB Expiry Date, the Principal Investigator is responsible for completing and submitting an HSREB Updated Approval Form in a timely fashion.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics Officer on behalf of Dr. Marcelo Kremenchuzky, HSREB Vice Chair

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Appendix C: Ethical approval for stent clinical study

Western University Health Science Research Ethics Board
HSREB Delegated Initial Approval Notice

Principal Investigator: Dr. Hassan Razvi
Department & Institution: Schulich School of Medicine and Dentistry/Surgery, Western University

Review Type: Delegated
HSREB File Number: 107941
Study Title: The Microbiome of Ureteric Stents
Sponsor: W. Garfield Weston Foundation

HSREB Initial Approval Date: June 27, 2016
HSREB Expiry Date: June 27, 2017

Documents Approved and/or Received for Information:

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The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the above named study, as of the HSREB Initial Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

[Redacted]

Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair

Ethics Officer: Erika Bauble __ Nicole Kaniki __ Grace Kelly __ Katelyn Harris __ Vikki Tran __ Karen Gaspad __

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