Characterization of the Urine Microbiome-Host Interaction

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Abstract

Background: The Human Microbiome is an area of increasing interest, both in and outside of the scientific community. While overall knowledge has increased exponentially, many aspects remain unclear, such as mechanisms of host-interaction and persistence.

Intracellular bacteria have previously been described in urothelial cells, but only as a mechanism for pathogen persistence in *E. coli*. Using the urinary microbiome as a model for the human microbiome, we set out to uncover, and better characterize the intracellular microbiome.

Methods: Participants without recent urinary tract infections or antibiotic use were enrolled in a cross-sectional study. We used several techniques to analyze voided urine samples from each participant to assess for intracellular bacteria. Imaging flow cytometry and volumetric flow cytometry were performed using antibodies against uroplakin III and Enterobacteriaceae common antigen (ECA). Scanning electron microscopy (SEM) also utilized anti-ECA antibody after lysing urothelial cells with sonication. Lastly, 16S rRNA metagenomic sequencing was performed with comparison to a reference library. Pairwise comparisons were conducted using the Wilcoxon rank sum test for continuous variables and the Fisher exact test for proportions. Microbiome analysis using the Qiime2 analysis package with alpha diversity calculated using four different methods: observed richness, Chao1, Shannon index and Simpson index. Beta diversity was calculated using both weighted UniFrac distances and Bray Curtis distances. Differential abundance was calculated using Analysis of Composition of Microbiomes with Bias Correction 2 (ANCOM-BC2).

Results: We recruited 20 participants of median age 38 (IQR 29-51) with diverse ethnic backgrounds between October-June 2022. Flow cytometry found evidence of intracellular

bacteria in 20/20 (100%) participants. Females had a higher number of urothelial cells in their urine (42 cells/μL vs 5 cells/μL, p=0.03) but the proportion of cells containing bacteria was similar at 13.9% (p=0.65). Visualizing with SEM, we found evidence of bacteria in 20/20 (100%) participants. Polymicrobial communities were visualized in a majority of participants (17/20, 85%) and bacteria were aggregated in an extracellular matrix in 20/20 (100%) participants. Metagenomics confirmed the presence of polymicrobial communities in 20/20 (100%) participants and found significant correlation between the concentration of DNA in the intracellular component of urine and the number of urothelial cells containing bacteria ($p<0.0001$). There were significant differences in alpha diversity, beta diversity, relative abundance, and differential abundance when the extracellular and intracellular urine components were compared.

Conclusion: We found evidence of a significant intracellular component of the urinary microbiome in healthy individuals. Bacteria were found in 14% of urothelial cells and were visualized together in polymicrobial communities, likely embedded within a biofilm-like structure. The intracellular component was had differing alpha diversity, beta diversity, relative abundance, and difference abundance compared to the extracellular. This large intracellular component of the microbiome explains how the urine microbiome is able to persist within the human urinary tract with its flushing. Sex-based variations were also identified with beta diversity and differential abundance differing significantly between the intracellular components of males and females.

This is the first-time intracellular bacteria have been described in the urine of healthy individuals. While there are many future implications, this study demonstrates that the method of

DNA extraction in microbiome analysis plays a massive role in any potential findings. Future studies on the human microbiome in any location, should evaluate for intracellular bacteria.

Preface

This thesis is an original work by Robert Benson Weyant. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “IBC-5 - Healthy Urine Microbiome”, No. Pro00124267, October 10th, 2022.

The experiments described were performed by myself and Dr. Carlos Cervera, with the exception of the 16S rRNA sequencing as described (section 2.7). This paper represents my original work and has not been published elsewhere.

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Abbreviations

ANCOM-BC2 - analysis of Composition of Microbiomes with Bias Correction 2

CAUTI – catheter-associated urinary tract infection

CDI – *Clostridioides difficile* infection

CFU – colony forming unit

ECA – Enterobacteriaceae common antigen

eDNA – extracellular deoxyribonucleic acid

EPS – extracellular polymeric substances

EQUC – enhanced quantitative urine culture

EtOH – ethanol

FACS – fluorescence-activated cell sorting

FMT – fecal microbiota transplantation

GI – gastrointestinal

GU – genitourinary

HMDS – hexamethyldisilazane

HMP – human microbiome project

IBD – inflammatory bowel disease

IBC – intracellular bacterial communities

IBS – irritable bowel syndrome

IC – interstitial cystitis

IQR – interquartile range

LPS – lipopolysaccharide

NGS – next generation sequencing

PBS – phosphate-buffered saline

RA – relative abundance

SCFAs – short-chain fatty acids

UPIII – uroplakin III

UPEC – uropathogenic *E. coli*

UTI – urinary tract infection

UUI – urgency urinary incontinence

1. Introduction

1.1 The human microbiome

The human microbiome is a vast and relatively new concept. Microbiota refers to the collection of micro-organisms themselves, whereas the microbiome consists of their collective genomes. We now know that in healthy or diseased states, microorganisms can be found on any mucosal surface or exposed area of the human body - whether its skin, the gastrointestinal (GI) tract, or genitourinary (GU) tract. These microbes include bacteria, viruses, archaea, as well as eukaryotes such as fungi and protozoa. Over the past decades our understanding of the human microbiome has expanded exponentially due to breakthroughs in sequencing technology and data processing. Not only are microbes found on almost every surface of humans, but the diversity therein is immense. Trillions of bacteria live on and inside humans, equaling human cells and forcing us to re-evaluate what it truly means to be human.[1,2]

Furthermore, these microbes do not live in isolation, they exist in an environment of constant interaction with each other, as well as host cells. Communication involves physical connections, messenger molecules, quorum sensing, and metabolic by-products. Far from benign, these interactions play important roles in various facets of our lives. Like a diverse rainforest, the balance of species diversity, and the presence or absence certain organisms is critical.

While dynamic throughout life, the initial microbiome was thought to be acquired through vertical transmission. Dogma was that humans were born sterile and this was supported by observational studies that found neonates delivered by cesarian section to have a different microbiome profile than those delivered vaginally.[3] Those delivered vaginally tend to have more vaginal flora such as *Lactobacillus*, *Prevotella* or *Sneathia* spp., while cesarian section leads to more skin flora such as *Staphylococcus, Corynebacterium* and *Propionibacterium* spp. Further studies have revealed that it is not that simple, with the neonatal microbiome being influenced by factors such as peripartum antibiotics, pregnancy complication and length of labour.[4] Along with this, increasing evidence has found bacteria in sites that were initially thought to be sterile such as the placenta and amniotic fluids, hypothesizing that bacterial acquisition may occur before birth.[5] Genetics are also thought to play a role and if the microbiome was entirely maternally transmitted, then mother and child would have identical microbiomes. In fact, monozygotic

twins have a more highly correlated microbiome than dizygotic, and both have more similarly related microbiomes than non-twins.[6]

The intestinal tract is home to the highest density of micro-organisms and most analysis to date on the human microbiome has been on the fecal microbiome due to its abundance, early discovery, and ease of collection. We owe most of our knowledge about the microbiome to breakthroughs in sequencing techniques such as next generation sequencing (NGS). NGS encompasses a variety of techniques and can either sequence an individual genome or the metagenome of multiple species, as in the case for microbiomes. Shotgun sequencing, a type of NGS, requires breaking down large strands of genetic material into shorter sequences which are reconstructed via overlapping segments before being referenced against a library of known sequences. Shotgun amplicon sequencing uses primers against specific sections, for example 16S ribosomal RNA, which are sequenced and used to quantify each taxon. The end result is typically a relative abundance (RA) graph. Other terms used in microbiome analysis include alpha diversity (within a single sample), and beta diversity (between samples), as well as various indexes such as the Shannon index (one of the most common ways diversity is measured).[7]

When discussing microbiomes, terms such as "healthy" or "dysbiosis" are frequently used, though the distinctions are not always clear. A healthy microbiome will change throughout an individual's life. As an example, diet can influence the microbiome and changing to a more carnivorous diet increases the relative amount of bile-tolerant microbes and decreases bacteria that breakdown plant polysaccharides.[8] Most bacteria in the fecal microbiome are either obligate or facultative anaerobes. The major genera of obligate anaerobes include: *Bacteroides, Bifidobacterium, Clostridium, Eubacterium, Fusobacterium*, *Peptococcus, Peptostreptococcus*, and *Ruminococcus* while the facultative anaerobes include *Escherichia, Enterobacter, Enterococcus, Klebsiella, Lactobacillus* and *Proteus.*[9]

The microbiome has been implicated in both health and disease. In the gastrointestinal (GI) tract it facilitates digestion by allowing for the breakdown of plant cell walls (non-digestible fibres to humans) as well endogenous intestinal mucous. Cellulose, which consists of β-1,4 linked glucan chains, cannot be digested by human enzymes such as amylases, sucrase and lactase. Plants also contain other complex carbohydrates such as pectins and xyloglucans. The whole of the microbiota produces hundreds of other enzymes such as glycoside hydrolases and polysaccharide

lyases that are able to break down the various structures of plant walls[10] and in return, mostly through anerobic digestion, it produces short chain fatty acids (SCFAs) and gas. SCFAs are fatty acids with 6 or less carbon atoms produced by the anaerobic metabolism of fibre and starches. Most common are acetate, propionate and butyrate, with butyrate being the main source of energy for colonocytes.[11] Beyond providing nutrition, they are associated with an improved gut barrier and decreased inflammation through interactions with regulatory T cells (Tregs), which help limit colonic inflammation. In a murine model, when given to microbiome-devoid mice, SCFAs helped upregulate Tregs and prevented the development of colitis.[12]

Many vitamins are also synthesized by gut bacteria, including vitamin K and B vitamins (biotin, cobalamin, folates, nicotinic acid, pantothenic acid, pyridoxine, riboflavin, and thiamine).[13] Essential for human survival, we depend on bacteria to provide us with these vitamins. For example, the antibiotic trimethoprim-sulfamethoxazole is effective because it inhibits bacterial folate production, leaving human cells unscathed.[14] In addition, most clinicians are aware that antibiotics will increase the international normalized ratio (INR), a measure of blood clotting, by decreasing vitamin K synthesis.

Homeostasis of the microbiome is important for infection prevention. *Clostridioides difficile*, an anaerobic spore forming bacteria, is the posterchild for GI tract microbiome dysbiosis. The bacteria, which can colonize the colon of healthy individuals, proliferates in the presence of dysbiosis. This commonly occurs in those with diarrheal illnesses and antibiotic use. When the healthy microbiome is displaced, *C. difficile* moves in to take its place and overgrowth can cause a disease known as pseudomembranous colitis, which can have significant morbidity and mortality. Treatment of *C. difficile* infection (CDI) is difficult, as it is a disease often caused by antibiotics and the treatment, more antibiotics, does not address the issue of dysbiosis, leading to high recurrence rates. For this reason, probiotics, and fecal microbiota transplantation (FMT) have been investigated as an alternative or adjunctive treatment with some success. Immediately following FMT, the recipient's microbiome appears similar to the donor's. Later, the two microbiomes diverge, but the recipient's microbiome remains in the healthy range for months.[15] FMT has also been shown to decrease the amount of antibiotic resistance genes in recipients for at least one year.[16]

The idea of ingesting human fecal matter to treat disease dates back to at least the 4th century, when Chinese literature described using it to treat diarrhea and food poisoning.[17] However, it would not be until 2013 when the first RCT was performed involving FMT. Stopped early due to efficacy (81% resolution vs 31%), the study looked at duodenal infusion of human feces to treat recurrent CDI compared to antibiotics.[18] Further studies have found similar efficacy for FMT delivered via other routes such as orally and current medical guidelines suggest using FMT for refractory cases of CDI.[19] In 2022 the first microbiome product, Rebyota™, was FDA approved for the prevention of recurrent CDI based off an RCT which showed a 70.6% cure rate at 8 weeks compared to 57.5% with placebo.[20] The product contains a "diverse set of micro-organisms" and is administered as a rectal solution.

Despite the development of FMT and its therapeutic uses, we are still far from understanding exactly how the intervention works on a cellular and molecular level. It not as simple as giving a *Lactobacillus* supplement because most studies that involve giving a single probiotic are not effective. It is something more than just a bacterial species, or even multiple bacterial species. Some theorize that commercial probiotics are missing key factors such as the metabolome, the sum of the metabolic products of the microbiota, as well as key viruses such as bacteriophages. Bacteriophages, which are estimated to outnumber bacteria in the GI tract by about four-fold, are thought to play a significant role in regulating biodiversity and facilitating gene transfer.[21]

There is a temporal association between the rise of antibiotic use and increasing prevalence of autoimmune diseases such as inflammatory bowel disease (IBD), type 1 diabetes mellitus (T1DM), multiple sclerosis (MS) and systemic lupus erythematous (SLE), leading some to theorize that disruption of the microbiome and subsequent dysbiosis is a factor. Through the development of gastric-associated lymphoid tissue (GALT), and balancing of Th1, Th2 and Th17 immune responses the microbiome plays an important role in the immune system.[22] Studies to elucidate the effect of the microbiome have been performed using germ-free mice models. Overall, these germ-free mice show a significant impairment of gastrointestinal lymphocyte function as well as a reduction in IgA, that can be reversed with microbial colonization.[23] *B. fragilis* contributes to CD4 T cell development and Th1 differentiation through production of polysaccharide A.[24] Natural killer (NK) cells are also affected by the microbiome, requiring type 1 interferon (INF-1) priming from monocytes, which is impaired in germ-free mice.[25] Th17 cells,

a class of immunomodulatory cells, secrete cytokines that cascade towards antimicrobial peptides, reinforcement of gut tight junctions and promotion of neutrophil recruitment via release of granulocyte colony stimulating factor (G-CSF). Several commensal bacteria, such as *Bifidobacterium adolescentis*, have been found to induce Th17 response.[26]

Reviewing the entirety of the interactions between the thousands of bacteria, the many signalling factors of the immune system and the hundreds of autoimmune diseases is outside the scope of this dissertation. However, we do know that patients with IBD have reduced alpha diversity compared to healthy individuals or even monozygotic twins without IBD.[27] This dysbiosis is predominantly due to a decrease in *Bacteroidetes* and *Firmicutes*, the two most predominant phyla in the intestinal microbiome, as well as increases in other phyla.[9,22] Correlation also exists between the degree of dysbiosis and disease activity. Despite this connection, studies looking at probiotics in autoimmune diseases (mostly IBD) have had limited success. One study which used several species of *Lactobacillus* and *Bifidobacterium* found some improvement in mild ulcerative colitis, but the results have not been translated to Crohn's disease or more severe colitis.[28] A meta-analysis on FMT for IBD showed variable efficacy,[29] and a small randomized clinical trial showed some improvement in endpoints in T1DM.[30]

Another area of active microbiome research is obesity. Obesity is heavily studied due to its prevalence in modern society and its myriad of associated health conditions. Due to the connection between food digestion and the microbiome, the gut microbiome is a logical place to investigate. In animal models, obese subjects have a greater proportion of *Firmicutes* with depletion of *Bacteroides*.[31] Transferring a specific *Bacteroides* species to obese mice results in a decrease in adiposity.[32] There have also been associations found with multiple other species such as *Akkermansia, Lactobacillus, Bifidobacterium* and *Enterococcus*. In humans, higher levels of microbial diversity seems to have a protective effect against obesity and again, a higher ratio of *Firmicutes* to *Bacteroides* seems to have a negative effect.[31] Interestingly, bariatric surgery increases levels of *Bacteroides* and works to correct dysbiosis.[32] Despite these connections to the microbiome, studies looking to intervene with probiotics have only shown modest effects.[33]

While most of our knowledge on the human microbiome comes from studying the gut microbiome, it does have its limitations. For one, a stool sample contains much more than just micro-organisms, there is undigested carbohydrates and fats as well as mucous and epithelial

cells.[34] Analysis of stool typically requires reconstituting the solid into a liquid suspension and sample collection can be difficult and intrusive.

1.2 The healthy urine microbiome and its relevance as a model

Urine has long fascinated scientists and physicians. Before the development of imaging techniques and phlebotomy, the only way to see inside the human body and glimpse into its function, was by assessing its metabolic by-products. In fact, the ancient word for urinalysis was uroscopy, a combination of the word urine and the Greek word skopeo, meaning to 'behold, contemplate, examine, inspect'.[35] Ancient Hindu cultures astutely recognized that some people had sweet urine that attracted ants, a disease know known as diabetes mellitus (Greek words for siphon and sweet).

Fortunately, our understanding of urine has progressed over the years beyond having to taste it, but there is still much we do not know. We know that urine is produced in the kidneys and transferred through the ureters to the bladder where it is stored until it is expelled through the urethra. Urine composition is >90% water with the rest being urea, organic and inorganic salts, and biologic material.[34] A typical adult will urinate about 1.5L of urine/day, depending on fluid intake. The concentration and composition of urine fluctuates over the course of the day and varies with dietary intake and exercise. Urine's high volume and ease of collection is what makes it a great model to study the microbiome.

For many years, the dogma was that urine was sterile in healthy individuals. The roots of this thinking go back to the 1800s with the pioneers of microbiology such as Louis Pasteur and sssJoseph Lister.[36] They noted that urine in a sealed container did not go cloudy and thus concluded it was aseptic. Intuitively, this makes sense as for micrsobes, urine is a relatively hostile growth medium. It is hypertonic, acidic, and salty, in addition the bladder flushes itself multiple times per day.[37] The first published mention of commensal bacteria in the urine came from 1956 when Edward Kass assessed urine samples from symptomatic and asymptomatic individuals and determined that 10^5 colony forming units (CFU) was the threshold at which urinary tract infection (UTI) symptoms appeared.[38] In 2012 an experiment comparing routes of urine collection showed that samples collected from suprapubic aspiration and transurethral catheter both contained bacteria, disproving that urine is sterile.[39] The same study found that

voided samples contained a mix of urinary and genital tract bacteria. Recently the term asymptomatic bacteriuria has grown in popularity to indicate the presence of bacteria in the urine, but the absence of symptoms of cystitis. The term is an artifact of historical urine culture methods, as we now know that every urine sample taken (even from healthy individuals) from birth until death will contain bacteria.

Initially, urine was tested for "sterility" using urine cultures. The standard urine culture is done by plating a small amount (1 μL) of urine onto MacConkey and blood agar, then incubating at 35°C for 24hr.[40] Designed to culture common uropathogens such as uropathogenic *E. coli* (UPEC), this ignores slower growing bacteria, those that require an anaerobic environment, and bacteria present in low CFU amounts ($<10^3$). More advanced techniques for assessing the urinary microbiome include NGS (discussed in section 1.1) or the enhanced quantitative urine culture (EQUC) which plates a larger amount of urine (100 μL) on a variety of media in diverse atmospheric conditions for twice as long (48 hours).[41] Compared to next generation sequencing (NGS) this has the advantage of showing viable bacteria, but it still doesn't capture the whole picture.

As mentioned, most of the scientific developments on the human microbiome came from the GI tract, where the presence of bacteria is more obvious. When the Human Microbiome Project (HMP) launched in 2008 it initially only assessed the GI tract. Later, it was expanded to assess five sites: gastrointestinal, oral, skin, nasal and urogenital.[9] The urogenital samples involved swabbing both the vagina and posterior cervical fornix. Urine was not included despite decades of knowledge that is it not sterile, possibly because of difficulty with culturing techniques, or it was thought that asymptomatic bacteriuria came from genitourinary (GU) contamination. The genomic sequencing of the HMP was done using 16S rRNA amplicon sequencing.

It has become more and more accepted that the urinary tract has a microbiome and that it is distinct from both the gut and genitourinary microbiomes[39] As in the gut microbiome, defining healthy or normal is a difficult task. There are many factors that influence a person's urinary microbiome. Variations exists between sexes in both composition and bacteria. For example, females have higher amounts of citrate but less calcium and oxalate, and males have more creatinine.[37] Menopausal females are disproportionally affected by UTIs, suggesting a hormonal effect. Estrogen has a protective effect on urothelial cells, it stimulates production of

antimicrobial peptides and reinforces the tight junctions between epithelial cells. Conversely, the same study found that the presence of estrogen increased UPEC internalization inside the epithelial cells.[42] Mouse models of menopause also have higher degrees of bacteriuria, more intracellular bacterial reservoirs, and aberrant immune responses. Findings, which were reversible with estrogen supplementation.[43]

As we age, so does our microbiome. One study that assessed a wide age range of females found that there were age-specific genera of bacteria including *Jonquetella, Parvimonas, Proteiniphilum,* and *Saccharofermentans* in females >70 years old – in addition to more standard genera such as *Lactobacillus.*[44] The method of urine collection can also have an effect on the microbial diversity that is detected. There are multiple methods including: first catch, midstream, transurethral catheterization, and suprapubic aspiration. The latter two methods are thought to get a more accurate representation of the urinary microbiome by avoiding the contamination of the genital microbiome.[39]

In females without UTIs the most abundant genera of bacteria include *Lactobacillus, Prevotella, Streptococcus* and *Gardnerella.*[45] Males also have an abundance of *Lactobacillus, Streptococcus* and *Prevotella*, but have less *Gardnerella* and more *Sneathia* spp.[46] Multiple factors determine what makes a bacterium a good urinary commensal. When looking at organisms that are culturable using standard techniques, some common microbial characteristics that predispose to asymptomatic bacteriuria are the ability to use urine as a substrate, de-novo synthesis of guanine, catabolism of malic acid and resistance to D-serine.[37]

Lactobacillus is the predominant genera in healthy females (*Lactobacillus crispatus* and *Lactobacillus iners*). Thes genus are thought to prevent infections through multiple methods: release of lactic acid, production of H_2O_2, biosurfactants and by aggregating with pathogens.[47] Females with recurrent UTIs may have more *E. coli* species and depletion of Lactobacilli. In-vivo experiments with *Lactobacillus* show that their metabolic by-products and their culture supernatant prevent the growth of UPEC and downregulates some virulence factors.[48]

There is increasing evidence that the urinary microbiome, like its fecal counterpart, is acquired prior to birth. Metagenomic studies have found that the placenta has its own microbiome, as does the uterus of the mother.[5,49] One study on the placental microbiome found that it was most

closely related to the oral microbiome, though comparisons were only made to sites utilized by the HMP and therefore the urinary tract was not included.[49]

Overall, the urinary microbiome is an extremely relevant model for assessing the complete human microbiome. For one, it has been historically overlooked due to misconceived notions of sterility. It is also a much simpler microbiome to assess than the fecal microbiome – given that samples come in a solution, and there is minimal extraneous solid material. As a model there are still significant gaps in our understanding. While we know some detail about the composition of the urinary microbiome, little is known about its structure. In addition, most of the studies cited above looked predominately at the female microbiome, due to the increased risk of UTIs, and little is known about the male microbiome. Knowledge about disease associations with the urinary microbiome are in their infancy as well.

1.3 Dysbiosis of the urine microbiome and related diseases

While the urinary microbiome may not aide in digestion like the fecal microbiome, it is by no means a benign system and plays crucial roles in the bladder immune system and urine production/metabolism. To best understand function of the health urinary microbiome, it is beneficial to see the diseases that are associated with its dysbiosis.

Urgency urine incontinence (UUI) – UUI is a prevalent but poorly understood condition. The disease is characterized by a strong need to urinate (urgency) that is accompanied by leakage of urine. The disease can be very burdensome and is thought to be due to abnormal neuromuscular signalling, resulting in an overactive detrusor muscle, the main muscle of the bladder.[50] However, detrusor overactivity is not present in all patients with UUI and treatment of it does not always result in symptom resolution. A connection between low microbiome diversity and UUI has been investigated due to overlapping symptoms with UTIs. Comparing females with and without UUI, one study found a significant difference in 14 different genera in patients with UUI. This included increases in *Sphingomonadales*, *Chitinophaga* and *Brevundimonas* spp. and decreases of *Mycobacterium*, *Nocardioides* and *Prevotella* spp. There was also a correlation between the severity of UUI symptoms and loss of alpha diversity.[50] Another study used 16s rRNA PCR as well as EQUC and found that patients with UUI had increased *Gardnerella*, decreased *Lactobacillus* and increased levels of 9 specific genera, however a completely

different 9 genera than in the previously mentioned study - demonstrating some of the difficulty with microbiome analysis. Of the *Lactobacillus* isolated, UUI patients had more *Lactobacillus gasseri* and the controls had more *Lactobacillus crispatus*.[51] In a step towards personalized medicine, solifenacin, a medication used to treat UUI was found to be more effective in patients with lower urinary microbiome diversity.[52]

Urolithiasis – Urolithiasis, the presence of calculi or stones in the urinary tract, is a prevalent and often painful condition with a lifetime prevalence of around 15%.[53] There are multiple different types of stones, all of which have different risk factors. Struvite stones (sometimes called infection stones) are produced by urease-containing bacteria such as *Proteus*.[54] Calcium oxalate stones, the most common type of urolithiasis, are caused by hyperoxaluria (too much oxalate in the urine).[55] *Oxalobacter*, part of the intestinal microbiome, catabolizes oxalate to produce ATP, thereby decreasing the oxalate absorbed and secreted by the kidney. Observational studies have found that patients with calcium oxalate stones have less *Oxalobacter* in their stool.[56] *Oxalobacter* is less common in females with recurrent UTIs, possibly due to multiple courses of antibiotics. Antibiotic use also shifts the urinary microbiome away from *Lactobacillus* and towards *Enterobacteriaceae*, which is also associated with stone formation.[57] There is also evidence that calcium oxalate stones are potentiated by the urine microbiome. In-vitro models show that *E. coli, K. pneumoniae, S. aureus* and *S. pneumoniae* all promote calcium oxalate crystallization and aggregation.[58] Stones can also be colonized by bacteria, potential serving as a nidus for the urinary microbiome or recurrent infections.[59]

Urothelial carcinoma – Many infections are known to be associated with cancer, for example *H. pylori*, human papillomavirus, Hepatitis B and C viruses, and in the bladder, *S. haematobium.*[60] As discussed, the microbiome plays an important role in modulating the immune system, and one of the jobs of the immune system is cancer protection. Colorectal inflammation in mouse models is associated with dysbiosis and colorectal cancer.[61] In humans the connection is not as clear but it remains an area of active research for both colorectal and urothelial carcinoma. In a small pilot study, patients with urothelial carcinoma were found to have higher amounts of *Streptococcus* spp. than those without.[62] Overall, the data is unclear, as a different study found no such association and a third found that bacterial richness was increased in urothelial cancer patients.[63,64]

Interstitial cystitis – Interstitial cystitis (IC) is a disease of unclear etiology. Symptoms include chronic pelvic or bladder pain with lower urinary tract symptoms (LUTS). Females are disproportionately affected compared to males. Being a diagnosis of exclusion, the disease is difficult to diagnose and study.[65] Females with IC have less genera and less overall diversity than healthy females.[66] Microbiome analysis has found decreased *Prevotella* and *Gardnerella* with increased *Enterococcus, Atopobium, Proteus* and *Cronobacter* spp. Interestingly, the same study also found increased *Lactobacillus* (not seen in other studies or other disease states).[67] A larger follow-up study did not see differences in relative abundances but there were differences in *Lactobacillus* (more *johnsonii* and *gasseri* in IC, but less *acidophilus*) and more uropathogens in the IC group.[68] Two other studies have been performed that have not found a relationship between IC and the microbiome.[69,70]

Urinary tract infections – A significant amount of urinary microbiome research has been done regarding its relationship with UTIs, and for good reason. UTIs are the 2nd most common type of treated infection and 50% of females experience at least one episode in their lifetime.[71] In medicine UTIs are divided into complicated and uncomplicated with complicated UTIs requiring a different approach to treatment. Some examples of complicated UTIs are those that occur in people with anatomical abnormalities, immunosuppression, post-surgical or in males. While uncomplicated UTIs (acute cystitis) are straightforward to treat (often requiring only a few days of antibiotics), a sizable percentage of individuals will go on to develop recurrent UTIs. Recurrent UTIs (discussed in more detail in section 1.5) are very prevalent and an estimated 25% of females go on to have a recurrent UTI within 6 months of treatment.[72] Risk factors include hormonal deficiencies, diabetes, nephrolithiasis, structural and functional bladder diseases, and antibiotic use.

The current model of urinary tract infection is the ascending infection model (outside of exceptions from surgery or devices such as catheters). In this model, bacteria from outside the urinary tract move up the urethra and take hold in the bladder, reproducing and leading to the symptoms of cystitis. Males are thought to be less susceptible to UTIs as the longer urethra makes it more difficult for bacteria to ascend. Infecting strains can often be found in vaginal and fecal cultures of patients with recurrent UTIs, and this has lent support to the ascending route of

infection, though this does not prove causation but rather that the patient is colonized with specific bacteria.[73]

Supplementing the ascending infection model is increasing evidence that the microbiome plays a significant role in infections. Continuing a common trend, as Lactobacilli decrease, the *E. coli* population increases.[71] Urinary microbiome is also home to many bacteriophages with one study sequencing the genomes of 181 bacterial species in the urinary microbiome and finding 226 phage sequences. It was also revealed that 86% of bacteria had a phage sequence with a viable *E. coli* lytic phage being cultivated.[74] In addition, there is some evidence for probiotics in UTIs, pointing towards UTIs as a disorder of the microbiome rather than solely urinary tract anatomy. Interestingly, there have been several case reports of FMT successfully treating recurrent UTIs, with larger studies ongoing.[75–77]

Realizing that UTIs are a disease of the microbiome would have large implications for our overall understanding and management of the disease. Treatment of UTIs typically involves antibiotics for 1-7 days (depending on severity and the antibiotic used), which may disrupt the urinary microbiome even further. As demonstrated in *C. difficile* colitis, the treatment of a microbiome disease with antibiotics can be extremely difficult. We also know that repeated courses of antibiotics leads to the development of resistant bacteria which is not only dangerous for the individual patient, but is becoming a global concern.[78]

1.4 Bacterial biofilms and human disease

Biofilms are aggregates of micro-organisms embedded in an extracellular matrix, typically on the surface of an object (organic or not). Communicating constantly, the microbes don't just live in proximity - each biofilm is a dynamic ecosystem with microbes working together and filling various roles within the community. An important characteristic of biofilms is that they have emergent properties and can behave differently than the sum of their parts.[79] This has significant implications for persistence, antimicrobial resistance, and virulence. Beyond being of interest from an ecological perspective, biofilms also have a key role in healthcare and medicine.

Structurally, biofilms are an organised collection of microbes surrounded by extracellular polymeric substances (EPS). Water, extracellular polysaccharides, extracellular proteins, and environmental DNA (eDNA) make up most of the EPS.[80] In *E. coli* and *Salmonella* spp. the EPS

consists of curli fibres (chains of peptides that are assembled into amyloid fibres) and the extracellular polysaccharide is cellulose.[81] Cellulose, a polymer of glucose that is linked by β-1,4 glycosidic bonds, is commonly thought of as a plant polysaccharide but can also be produced by bacteria.[82] eDNA, a conversed component of biofilms that is woven into a mesh-like structure, has been described in UPEC bacteria and is essential for their biofilm growth and stability.[82] In fact, eDNA is a therapeutic target in cystic fibrosis, where inhaled DNase is used reduce the viscosity of pulmonary secretions and biofilms. EPS composition also depends on the type of bacteria in the biofilm, with *Vibrio cholerae* and *Bacillus subtilis* making different extracellular proteins and polysaccharides than the aforementioned *E. coli.*[83] Biofilms can be analogous to multicellular organisms with the ability to undergo differentiation and have a life cycle of their own with different protein expression.[84]

Collectively, the micro-organisms and EPS serve many functions. Biofilms are defensive mechanisms that protect not only against mechanical stress but also other forces such as desiccation, with the high water component and dissolved proteins/polysaccharides acting as a gel. Secreted digestive enzymes and polysaccharides also afford biofilms the ability to adhere and persist on surfaces such as prosthetic implanted material. Biofilms can also confer significant antimicrobial resistance, one such way is the EPS providing a physical diffusion barrier.[85] Horizontal gene transfer and eDNA can pass along resistance genes to other bacteria in the biofilms. Other methods of antimicrobial resistance include enzymatic degradation and reactions with siderophores. Many cells in biofilms are in stationary growth, conferring resistance to antibiotics targeting replication.[86] All these methods can decrease the effective concentration of antimicrobials below lethal limits to potentially select for resistant bacteria.

Biofilms are commonly polymicrobial. In fact, the first multi-species biofilms were noticed by Antonie van Leeuwenhoek in the late 1600s, studying the bacterial plaques of his own teeth.[87] Many people who were never explicitly taught about biofilms are aware of this connection between dental plaques and carries. However, creating an extracellular matrix comes with a cost, so being polymicrobial must have a benefit. Like a multicellular organism, different bacteria in biofilms can fill different niches and play distinct roles. Aerobes trend to be closer to the surface, and anaerobes deeper down. Some bacteria also tolerate different pHs within the biofilm, and

others metabolise diverse chemicals such as nitrites.[79] Even single species of bacteria will develop different phenotypes within a biofilm.[88]

While biofilms are ubiquitous in nature, they may be best known for their role in nosocomial infections. Biofilms may form on any indwelling medical device such as intravenous or transurethral catheters, prosthetic implants such as heart valves or joint replacements. Outside of prosthetic material, biofilms are implicated in cystic fibrosis, endocarditis and prostatitis.[89] Their presence can lead to infections that are almost impossible to treat with antibiotics alone. In the bladder biofilms can form on transurethral (urinary) catheters, which are often used for urinary retention, and cause catheter-associated UTIs (CAUTIs), which are responsible for 40% of nosocomial infections.[90] The urinary catheter acts both as a nidus for biofilm formation and damages the urothelial mucosa upon insertion. *E. coli* and *P. mirabilis* are the most common causes, but CAUTIs can also be caused by *Enterococcus* spp., *Klebsiella* and others.[90] Similar to the protective effects of a healthy microbiome, one in-vitro study incubated catheters with colicin-producing *E. coli* and found that it protected the catheter from colicin-susceptible strains of UPEC.[91]

Outside of prosthetic devices, biofilms occur naturally within the body and have been described in both the gastrointestinal and urinary tracts. In the intestine, microbes must be in close contact with epithelial cells to interact and share metabolites. Commensals such as *Lactobacillus rhamnosus* bind to mucin, an abundant glycoprotein that makes up most of the mucosa. While biofilms of *Lactobacillus* have not yet been described in humans,[92] they have been described in murine and avian animal models.[93] Part of the difficulty describing the physical structure of the human microbiome is that most of our data points come from feces or rectal swabs which is far removed from structures such as the stomach and small intestine. A 1983 study looked at several sudden-death victims and found that bacteria were present below the mucin layer and that within the layer there was a 'complex microbial structure'.[94]

Microbiome dysbiosis can also involve biofilms. In a study that compared inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and healthy control patients, it was found that most patients with IBD and self-limiting colitis had high densities of biofilms, whereas only 35% of the healthy patients did. They also found that the density of intestinal biofilms was 100x

higher in IBD compared to IBS or controls. *B. fragilis* was responsible for most of the IBD biofilm mass but a minority of the biofilm mass in other groups.[95]

Due to their hardiness, assessment of the bacteria within biofilms is difficult. However, there is value in doing so to better understand and diagnose infections on prosthetic devices. Many different techniques have been used such as physical disruption, chemical dissolution, and sonication, but there is currently no universally accepted method.[96] Sonication, a technique using high frequency sound waves to disrupt biofilms, is the most commonly used technique and multiple studies have found increased microbial yield when the technique is applied to ex-vivo prosthetic joints and urinary catheters.[97,98] Despite this, most if not all studies assessing the urine microbiome do not use sonication or any other biofilm extraction technique.[45,51]

1.5 Intracellular bacteria and disease recurrence

UTIs are an extremely common infection and recurrence occurs in 25% of females within 6 months of treatment.[72] Seemingly going against our classical understanding of immunology, where once someone develops and then cures an infection, the body's immunity prevents that infection from occurring again. While people with anatomical or functional defects in the urinary tract (ex. neurogenic bladder in spinal cord injuries) can be predisposed to recurrent UTIs, in most people, there is no clear pre-disposing factor.

To learn how bacteria can establish themselves and create recurrent infections, it is important to describe the bladder immune response. A substantial portion of the bladder's immune response is through innate immunity rather than adaptive. TLR4, which responds to lipopolysaccharides (LPS), has a vital role in this innate immune response. TLR4 enacts an immune response through expression of IL-6 and IL-8, a neutrophil chemoattractant. Low IL-8 receptor expression is associated with pyelonephritis in humans, and UTIs in mice.[99] Mice with *TLR4* mutations are less responsive to LPS and more susceptible to UTIs.[100] Associations have also been made between children with asymptomatic bacteriuria and lower TLR4 expression.[101] In addition to LPS, type 1 pili may also stimulate TLR4 through FimH, the adhesion portion of the pili.[102] More than just *TLR4*, *TLR5* knockout mice (responds to flagellin) have higher bacterial loads in the bladder.[103] Many other factors of the immune system such as G-CSF, plasminogen activator inhibitor type 1 (PAI-1) and IL-17 also play a role.[99]

A key distinction to make in recurrent UTIs is relapse vs reinfection. Relapse is recurrence with the same organism, typically within a brief period of time. Reinfection is a new episode of infection, caused by a different bacterium. Recurrent UTIs commonly are relapses with the same bacteria, suggesting colonization either within or outside the bladder. Restriction fragment length polymorphism (RFLP) analysis done in 1995 in recurrent UTI patients found that the *E. coli* strains re-infecting patients were identical and were often found in the rectum prior to causing a UTI. They concluded that reinfection occurs from colonic strains.[104] A weakness of the ascending infection model is that attempts to sterilize the area around the urethra have not led to decreased UTIs. A 1985 study compared using a perineal topical disinfectant to oral antibiotics to prevent UTI recurrence and found that the topical disinfectant was ineffective,[105] implying that a reservoir exists within the urinary tract.

Further research into UTIs has found that bladder epithelial cells can harbour pathogenic bacteria. The most studied of these bacteria are uropathogenic *E. coli* (UPEC). While most of the research on intracellular bladder bacteria has been on UPEC, there is also evidence for several other species. *Staphylococcus saprophyticus* has been described invading urothelial carcinoma cells, while *Klebsiella pneumoniae* and *Salmonella enterica* are also capable of becoming intracellular.[106–108] Bacteria have developed many virulence factors in order to establish infections in the bladder including: adhesins, hemolysin and siderophores. One of the most important virulence factors for UPEC are its fimbriae. UPEC has two types of fimbriae to facilitate adhesion: type 1 pili adhere to glycosylated uroplakin on the luminal surface of the bladder while P-pili bind globoseries glycolipids on kidney epithelial cells.[109]

Two studies in 2007 investigated the significance of UPEC intracellular bacterial communities (IBCs) in acute cystitis. The first compared females with acute cystitis and those with asymptomatic bacteriuria using electron microscopy and immunofluorescence. IBCs were seen in 18% of the females with cystitis and 0% the asymptomatic patients.[110] The second study collected 18 UPEC isolates from females who had a variety of clinical syndromes such as asymptomatic bacteriuria, acute cystitis, recurrent cystitis, and pyelonephritis and inoculated them into mice. They found that 15 of the 18 samples formed IBCs, and that samples from all clinical syndromes were able to form IBCs, though those with acute cystitis formed smaller and fewer IBCs.[111]

IBC formation starts with UPEC cells attaching to bladder epithelial cells. There they take advantage of the cell's fusiform vesicles. These vesicles, which are normally used to regulate bladder area in the presence or absence of urine, are exploited by the bacteria to endocytose inside the epithelial cells where they can persist inside the bladder and escape voiding/elimination. The vesicles are cAMP dependent and exocytosis of the bacteria can be induced by giving medication to upregulate intracellular cAMP.[106] Inside the bladder endothelium, UPEC may be kept quiescent by the presence of intracellular actin, however upon undergoing terminal differentiation, the epithelial cell reduces its actin and UPEC may rapidly reproduce and create more intracellular pods.[112] For more efficient packing and division, the cells change from their standard rod shape to cocci and form biofilm-like structures where they are protected from flushing, host immune defences, as well as antibiotics.[113]

Being intracellular affords UPEC the ability to evade the immune system through several methods.[114] They can suppress NF-κB activity - subsequently inducing epithelial cell apoptosis, downregulate expression of IL-6 and IL-8, as well as attenuate neutrophil responses through multiple mechanisms.[99,115] Of course, the development of biofilm-like IBCs is critical to persistence, here they can avoid the immune system and continue propagation, leading to recurrent infections (biofilms discuss in section 1.4). Murine models of recurrent UTIs show that bacteria can endure for months and avoid the host immune system, as well as systemic antibiotics.[116] In one study, treatment with trimethoprim-sulfamethoxazole (a first-line antibiotic for UTIs) cleared the fecal reservoir acutely after 3 days, but 10 days of treatment was required to eradicate fecal colonization and decrease UTI recurrence. The bladder reservoir persisted after the 10 days of treatment, twice as long as a standard course of antibiotics for a UTI.[116] Once established, IBCs propagate through epithelial cell exfoliation or apoptosis, allowing UPEC to re-infect either other epithelial cells or the transitional epithelial below the superficial bladder epithelial cells.

Type 1 pili, previously thought of as an external attachment organelle, are essential for biofilm development.[117] When type 1 pili expression is downregulated, the bacteria are unable to form IBCs and bacterial burden is decreased.[118] Lactobacilli metabolic by-products can downregulate the promotor activity of type 1 and type P fimbriae and inhibit UPEC growth in-vitro.[48] If we think of UTIs as a disease of the microbiome, then it makes sense to attempt to treat the

dysbiosis. However, trials looking to replenish *Lactobacillus* orally[119] and intravaginally[120,121] have had limited success. In one study, females given *Lactobacillus* vaginal suppositories for secondary UTI prophylaxis after antibiotics were found to have a recurrence rate decreased from 47% to 21%.[121] A 2013 meta-analysis looked at 5 studies (both oral and vaginal suppositories) and found no difference in overall rUTIs, but sensitivity analysis of the 2 studies which looked specifically at probiotic strains (*Lactobacillus crispatus, rhamnosus* and *fermentum*) found a significant difference (RR=0.51) with NNT of 7.[122] Beyond *Lactobacillus, E. coli* has also been used to target dysbiosis. In patients with neurogenic bladder inoculation with a non-UPEC strain of *E. coli* was done in an effort to induce colonization and prevent recurrent UTIs. Colonization occurred successfully in over half of the patients, but it only lasted for a mean of 12 months. Those that were successfully colonized had a reduction in UTI frequency (3.1 UTIs/year to 0 UTIs/year).[123]

Not just a phenomenon of the urothelial epithelium, there is evidence for the involvement of intracellular bacteria and biofilms in otitis media, tonsilitis and cystic fibrosis.[124] Initially, the understanding of biofilms was that they had to be attached to an exterior surface but as we learn about the increasing complexity of biofilms, this is being questioned.[124] For all intents and purposes, the IBCs described inside urothelial cells behave as biofilms: they group together in close proximity, develop different phenotypes through quorum sensing, and contribute to persistence and resistance of antibiotics.

1.6 Hypothesis

While there is evidence showing that recurrent UTIs result from intracellular bacteria and dysbiosis of the urinary microbiome, we do not know much about the structure of the urinary microbiome, and if commensals are also able to form IBCs. To the best of our knowledge no one has described intracellular bacterial communities of non-pathogenic bacteria and the normal urinary microbiome.

We hypothesize that the current understanding of the urinary microbiome significantly underestimates the importance and scale of intracellular bacteria and IBCs. We expect that not only will bacteria be present in the urine of healthy individuals, but so will intracellular bacteria. These intracellular bacteria will have a distinct microbiologic profile from extracellular bacteria

and in addition, they will form a sizable portion of the urinary microbiome. Symbiosis of intracellular bacteria with human urothelial cells will likely require the presence of intracellular biofilms or biofilm-like structures, which we aim to visualize with SEM. Lastly, we predict that the microbiomes of males and females will have differences in the relative and absolute abundance of individual bacteria taxa. We aim to better characterize the intracellular component of the urinary microbiome through 3 main efforts.

1. Sonication of urine samples to disrupt the urothelial cell membrane prior to viewing with the IBCs with electron microscopy.
2. Using imaging flow cytometry to estimate the percentage of exfoliated urothelial cells that contain bacteria.
3. Comparing the urinary microbiome of acellular urine to the exfoliated urine cell pellet.

2. Methods

2.1 Study design

This was a cross-sectional study of participants without history of recurrent UTIs. Subjects were recruited by word of mouth and consented to enrol in the study (Research Ethics Board ID Pro00124267). A conscious effort was made to have a sex and race-diverse study group, with at least 50% females and 40% non-white participants. To select for healthy volunteers, subjects filled out a screening questionnaire stating that they were not currently on antibiotics and that they had not been diagnosed with a UTI in the past 6 months. Background data was collected on age of menarche and menopause, as well as whether the subject had ever been diagnosed with a UTI (**Supplemental Figure 1**).

2.2 Sample collection

Once enrolled, subjects were given sterile urine collection containers and asked to provide 300 mL of voided urine. Urine was randomly sampled and not midstream or catheter-collected for patient convenience. Some subjects provided samples over multiple days, but samples were always processed on the same day as collection.

2.3 Urine sample processing

The 300 mL urine sample was divided into six aliquots of 50 mL each. One aliquot was centrifuged at 300 x *g* and the resulting supernatant and cell pellet were collected in 2 mL PCR-clean tubes and stored at -80° C until processing for metagenomic analysis. The other five 50 mL aliquots were centrifuged at 300 x *g*, the cell pellet washed in phosphate buffer saline (PBS) and transferred to a 15 mL conical tube and centrifuged again at 300 x *g*. The resulting cell pellet was mixed with 1 mL cell culture freezing media with dimethyl sulfoxide (DMSO) (Millipore-Sigma, USA) and stored in cryogenic vials (Corning, USA) at -80° C until processing.

2.4 Imaging flow cytometry

We thawed one cryopreserved vial and washed the pellet with PBS first and 5% flow cytometry staining buffer (FACS) afterwards. Cells were then incubated for 30 minutes with a viability/cytotoxicity stain (LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, for 633 or 635

nm excitation, Invitrogen). Cell fixation and permeabilization was done using the Foxp3/Transcription Factor Staining Buffer Set (Invitrogen™, USA) following the manufacturer's instructions. We used IgG rabbit anti-uroplakin III (anti-UPIII) antibody (Invitrogen #MA5-16407, USA) and IgG mouse anti-Enterobacteriaceae common antigen (anti-ECA) antibody (Invitrogen #MA5-33256, USA) as primary antibodies, using a dilution of 1:200 for both antibodies and incubation in ice for 30 minutes.

After re-permeabilization, we added donkey anti-rabbit AF488 (Thermofisher #A21206, USA) and donkey anti-mouse AF647 (Thermofisher #A31571, USA), at a dilution of 1:170 and 1:200, respectively, and incubated in ice for 30 minutes. Cells were then washed in 5% FACS buffer twice and read within 24 hours. Cells were visualized and quantified using an Amnis® ImageStream®^{X} Mk II imaging flow cytometer.

2.5 Volumetric flow cytometry

We used volumetric flow cytometry to quantify the number of urothelial cells per volume of urine. Similarly to the imaging flow cytometry, we thawed one cryopreserved vial (equivalent to 50 mL of urine). The cells were washed with 5% FACS buffer, fixed, and incubated with primary antibody IgG rabbit anti-UPIII antibody (Invitrogen #MA5-16407) first, and donkey anti-rabbit antibody AF488 (Thermofisher #A21206) afterwards. Urothelial cells were quantified using an Attune™ NxT benchtop digital flow cytometer.

2.6 Scanning electron microscopy

One cryopreserved aliquot was thawed and washed with PBS. The cell pellet was suspended in 0.5-1 mL of PBS in a 1.5 mL microcentrifuge tube and was sonicated using a 42KHz and 35W ultrasonic cleaner (Model HC-80, Huatian Huichuang Technology, China) for 5 minutes in a water bath. The pellet was resuspended in 500 µL of PBS and then incubated for 30 minutes with anti-ECA antibody (Invitrogen #MA5-33256) in a 1:200 dilution. Samples were centrifuged and then resuspended in 500 µL of PBS solution before incubating for 30 minutes with 18nm Colloidal Gold-AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson Immunolab, US) at a dilution of 1:20.

The resulting pellet was fixed in 1 mL of glutaraldehyde 2% in 0.1M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, US), at 4° C for 24 h. Following fixation in glutaraldehyde, the pellet was fixed in osmium tetroxide 2% aqueous solution for 30 minutes.

The pellet was then washed twice using sodium cacodylate trihydrate (Sigma Aldrich Canada). Dehydration was done by increasing concentrations of ethanol (EtOH) and ethanol-hexamethyldisilazane (HMDS) as follows: 50% EtOH for 15 minutes, 70% EtOH for 15 minutes, 90% EtOH for 15 minutes, 100% EtOH for 10 minutes (four washes), EtOH:HMDS in a 75:25 ratio for 15 minutes, EtOH:HMDS 50:50 for 15 minutes, EtOH:HMDS 25:75 for 15 minutes and 100% HMDS for 15 minutes (two washes)[125]. The supernatant was discarded, and the pellet left to dry out overnight in the fume hood.

The dehydrated material was then mounted onto an ultra-thin carbon tab on an aluminium standard pin stub SEM mount with slotted 1/2" head (Electron Microscopy Sciences, US). The samples were visualized using a Hitachi S4800 FESEM.

Samples were screened under SEM visualization to look for bacteria. Both morphology (i.e. cocci and rods) and size (~0.5-2um) were used to screen for bacteria. Aggregates of bacteria were photographed and determined to be possibly monomicrobial if only one type of morphology was seen, or polymicrobial if both rods and cocci were seen.

2.7 Microbiome analysis

2.7.1 Sample preparation

In preparation, 50 ml of urine sample was centrifuged at 600 x *g* for 10 minutes. The sample was divided into two components, the cell pellet, and the cell-free sample, composed of 1mL of supernatant. With the cell pellets, we proceeded to sonication at 42 kHz for 5 minutes and DNA extraction using a commercial kit targeting bacteria in biofilms (DNeasy® PowerBiofilm® kit, Qiagen, Germany). For cell-free samples, we performed DNA extraction using a different commercial kit (ZymoBIOMICS DNA Miniprep Kit, Zymo Research, US). We measured the concentration of bacterial DNA in each sample using a Denovix DS-11 FX+ Spectrophotometer/Fluorometer. The extracted DNA from both cell-free and cell pellet samples

was shipped to the Centre for the Analysis of Genome Evolution & Function (CAGEF) at the University of Toronto.

2.7.2 16S rRNA gene sequencing

The V4 hypervariable region of the 16S rRNA gene was amplified using uniquely barcoded 515F (forward) and 806R (reverse) sequencing primers to allow for multiplexing.[126] Amplification reactions were performed using 12.5 µl of KAPA2G Robust HotStart ReadyMix (KAPA Biosystems), 1.5 µL of 10 µM forward and reverse primers, 7.5 µL of sterile water and 2 µL of DNA. The V4 region was amplified by cycling the reaction at 95°C for 3 minutes, 28x cycles of 95°C for 15 seconds, 50°C for 15 seconds and 72°C for 15 seconds, followed by a 5-minute 72°C extension. All amplification reactions were done in triplicate to reduce amplification bias, pooled, and checked on a 1% agarose Tris-borate-EDTA (TBE) gel. Pooled triplicates were quantified using PicoGreen and combined by even concentrations. The library was then purified using Ampure XP beads and loaded on to the Illumina MiSeq for sequencing (Illumina, San Diego, CA). Sequencing was performed using the V2 (150bp x 2) chemistry. A single-species (*Pseudomonas aeruginosa* DNA), a mock community (Zymo Microbial Community DNA Standard D6305), and a template-free negative control were included in the sequencing run.

2.7.3 Analysis of the bacterial microbiome

The Qiime2 analysis package was used for sequence analysis, with the following functions accessed from within the Qiime2 package.[127] The quality of the sequencing run was first examined using FastQC and MultiQC.[128,129] Cut adapt was used to remove sequences with high errors rates.[128] Paired-end sequences were assembled, and quality trimmed using vsearch –fastq_mergepairs with a –fastq_truncqual set at 2, a maxee set at 1, and minimum and maximum assemble lengths set at 250 and 255 (+2 and -3 base pairs from the expected sequence length of 253bp).[129,130] Assembled sequences were subjected to an additional filtering step, utilizing the quality-filter function in Qiime2. The resulting high-quality data was then processed following the deblur pipeline. Sequences were clustered into Amplicon Sequence Variant (ASV) groups and singleton sequences were removed. Taxonomy assignment was executed using the Qiime2 classify-hybrid-vsearch-sklearn function and the Average ReadyToWear trained Silva database

version 138.1[131,132]. ASVs with an abundance less than 0.01% were removed to reduce the potential for observing bleed-through ASVs, and ASVs identified as contaminating chloroplast or mitochondria were removed. A phylogenetic tree was created using the SEPP function available through Qiime2.[133]

2.8 Statistical analysis

Numerical analyses were performed using STATA V13 (StataCorp, College Station, TX) and microbiome analysis using the Qiime2 analysis package as described above. Variables were described as medians with interquartile ranges (IQR). Pairwise comparisons were conducted using the Wilcoxon rank sum test for continuous variables and the Fisher exact test for proportions. Alpha diversity was calculated with four different methods: observed richness, Chao1, Shannon index and Simpson index. Beta diversity was calculated using both weighted UniFrac distances and Bray Curtis distances, with significance tested via ADONIS. Differential abundance was calculated using Analysis of Composition of Microbiomes with Bias Correction 2 (ANCOM-BC2).[134]

3. Results

3.1 Demographics

Between October and June 2022, urine samples were collected from 20 participants, with a median age of 38 years (IQR 29-51) and predominance of females (12/20, 60%). Seven (35%) of the participants had previously been diagnosed with a UTI (6 females [50%] and 1 male [13%], p=0.09). None of the participants had been diagnosed with a UTI in the past 6 months or were on antibiotics at the time of sample collection. Ethnicity was as follows: 12 (60%) White, 3 (15%) Hispanic, 2 (10%) Filipino, 1 (5%) Black, 1 (5%) Arabic and 1 (5%) Chinese. For females, median age of menarche was 13 years (n=12, IQR 12-14) and for menopause it was 50 years (n=3, IQR 48-50).

3.2 Flow cytometry

Participants had a median urine cell concentration of 16.80 cells/µL (IQR 3.74-63.24), with 41.68 cells/µL in females and 4.97 cells/µL in males (p=0.03). Within these urothelial cells, imaging flow cytometry was able to visualize and quantify intracellular bacteria in 100% of subjects. The median concentration of urothelial cells containing intracellular bacterial communities (IBCs) was 1.54 cells with IBCs/µL (IQR 0.26-17.07), with 0.61 for males and 4.89 in females (p=0.03). This translated to a median of 13.9% (IQR 4.18-29.09) of urothelial cells containing bacteria. The proportion was similar between sexes with 12.6% in females and 15.7% in males (p=0.65).

3.3 Scanning electron microscopy

Presence of structures compatible with bacteria were found in processed urine pellet specimens of all participants (20/20, 100%) (**Figure 1**). Looking at the morphology of bacteria we found structures compatible with bacilli, measuring around 0.5-1.0 µm in width by 1.0-4.0 µm in length, in 19/20 (95%) participants. Structures compatible with cocci, measuring around 0.5-1.0 µm in diameter, were found in 17/20 (85%) participants. Combining the two together, we visualized structures consistent with polymicrobial communities in 16/20 (80%) participants (**Figure 2**). Structurally, we found evidence of bacteria embedded in a polymeric extracellular matrix in 20/20 (100%) of participants.

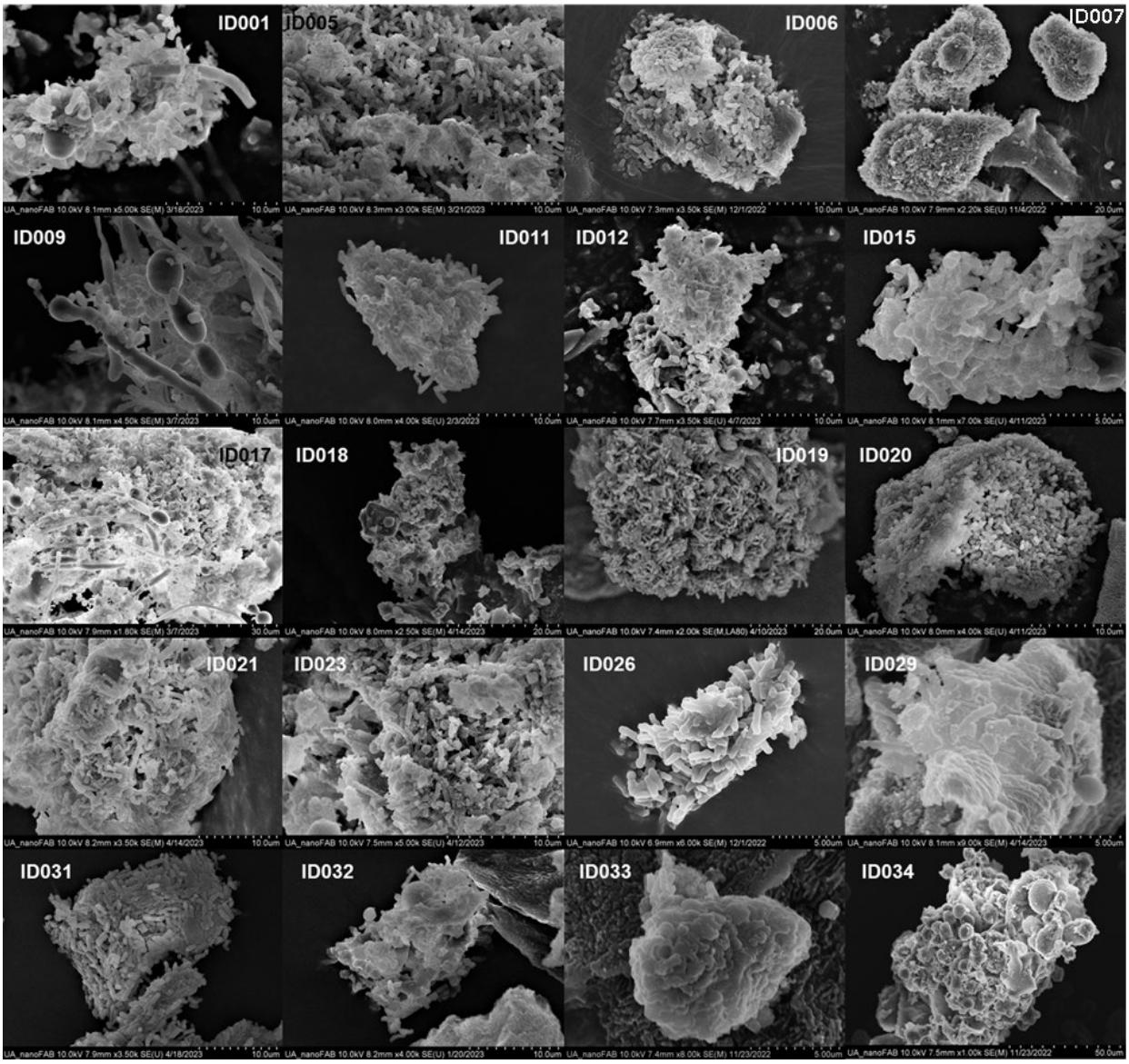

Figure 1. Example scanning electron microscope (SEM) photos of bacterial aggregates from each subject. A gold antibody was used to make Enterobacteriaceae more visible. Presence of both cocci and bacilli can be seem in all subjects and the bacteria appear to be attached to biofilm-like surfaces.

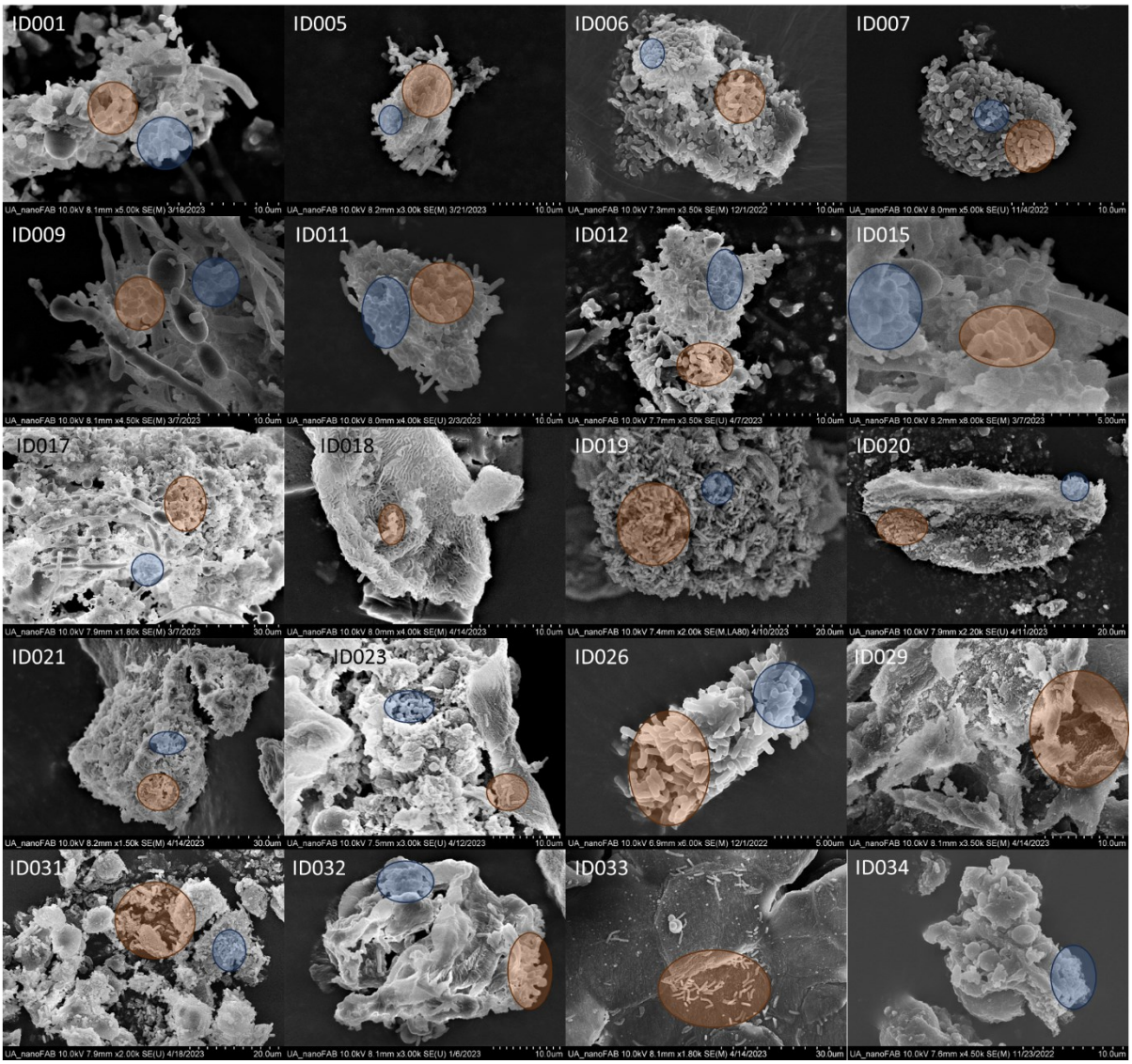

Figure 2. Example scanning electron microscope (SEM) photos of bacterial aggregates from each subject demonstrating either the presence of bacilli (orange) or cocci (blue), or both.

3.4 Microbiome analysis

We found significant correlation between the concentration of DNA obtained from cell pellets and the number of urothelial cells containing bacteria ($p<0.0001$) but not with the cell-free samples ($p=0.78$) (**Figure 3**).

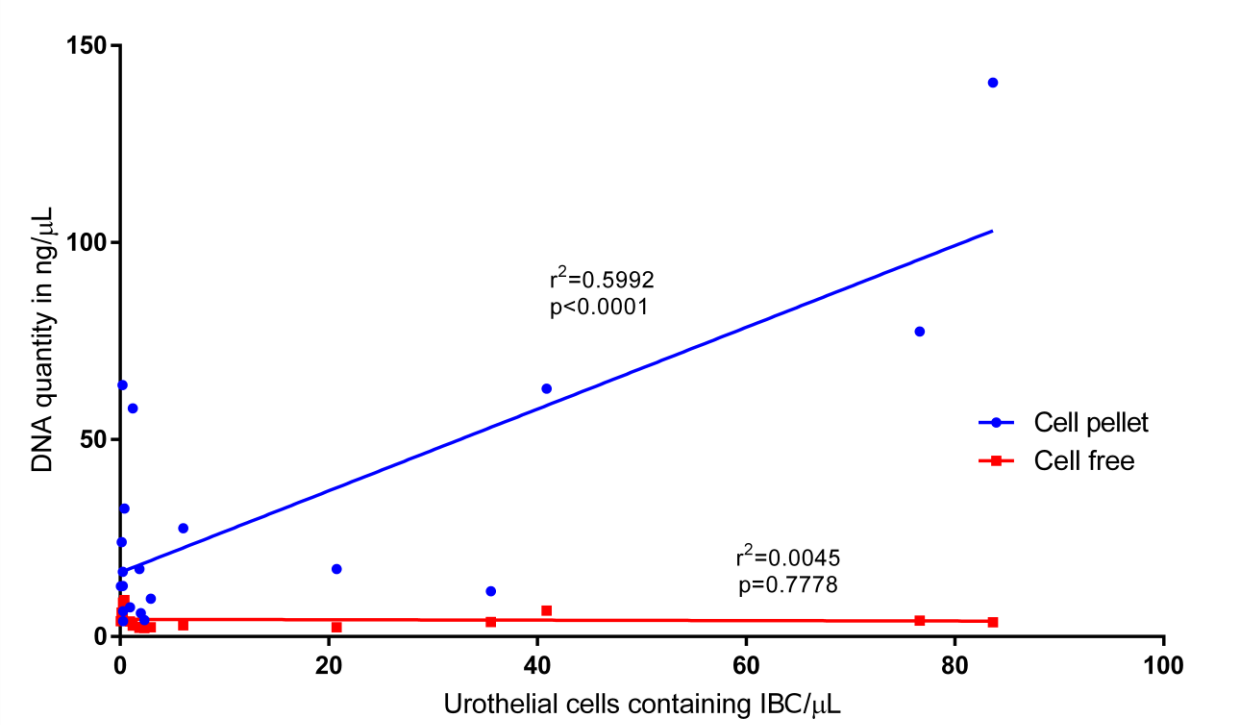

Figure 3. A plot of number of urothelial cells containing intracellular bacterial communities versus the quantity of bacterial DNA that was extracted from the sample. There is a strong correction when looking at the sonicated cell pellet, but there was no correlation with the cell-free DNA.

Metagenomic analysis revealed multiple differences between the cell-free and cell pellet samples. Cell pellet samples (intracellular component) overall had higher sequencing coverage, indicating a larger amount of DNA, while the cell-free samples had less sequencing coverage and a relatively higher amount of contaminant DNA (**Figure 4**). Cell-free samples had a much higher relative abundance of *Acinetobacter* while the pellet samples had more, among other, *Peptoniphilus, Streptococcus, Finegoldia* and *Staphylococcus* (**Figure 5**).

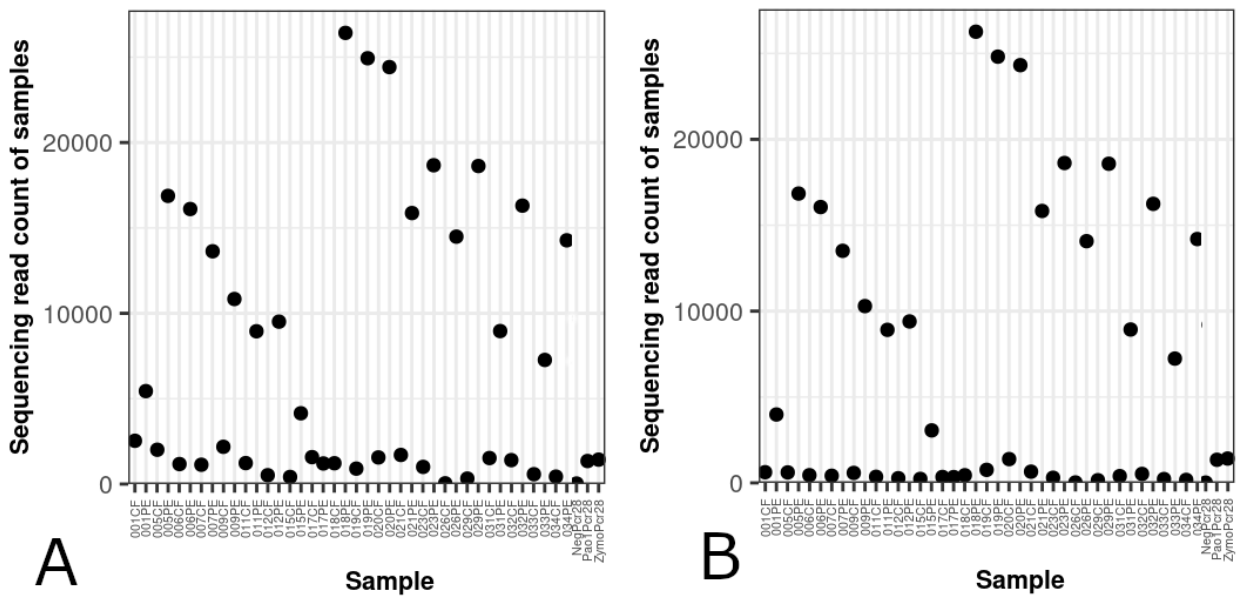

Figure 4. Sequencing depth of samples pre (A) and post (B) filtering of contaminants. The cell-free samples have relatively less sample coverage and a therefore a higher proportion of contaminant DNA.

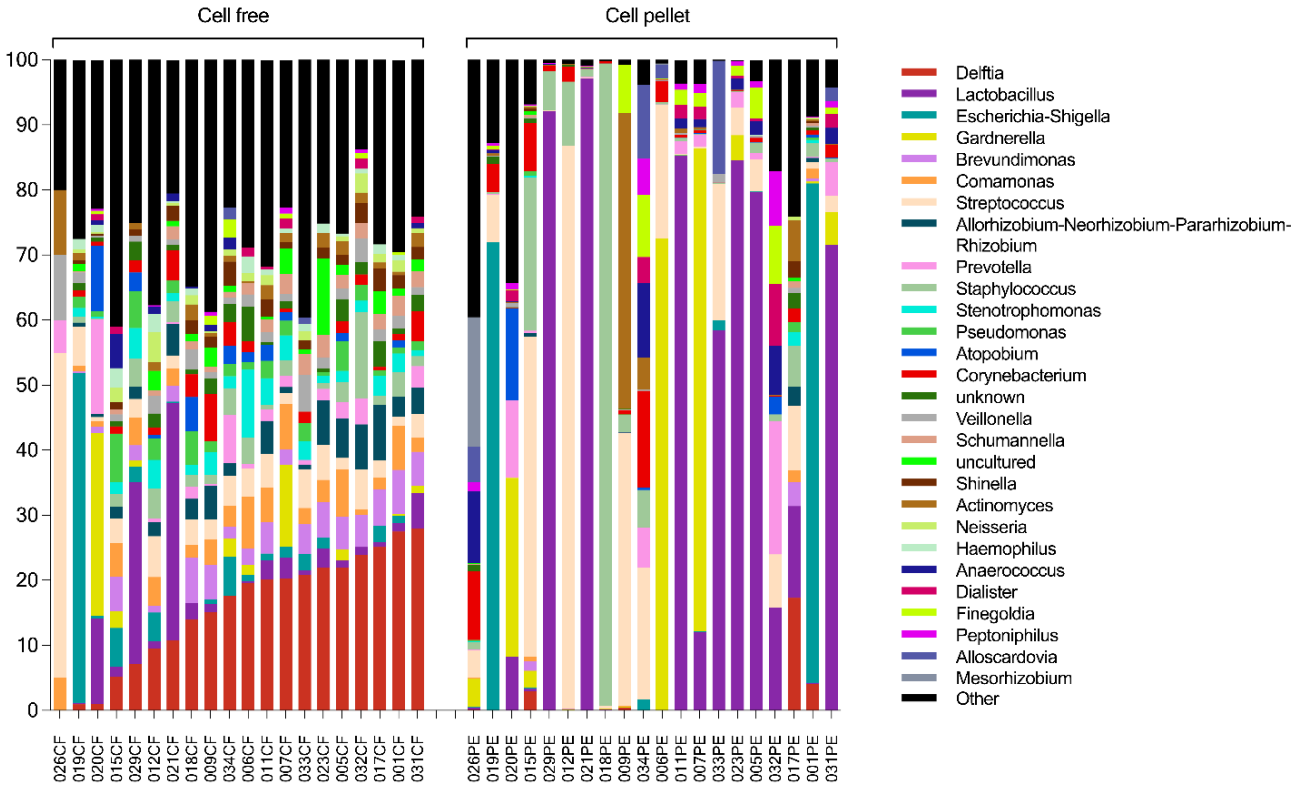

Figure 5. Relative abundances of genera of the cell-free and cell pellet samples. Subjects were sorted according to likeness of the cell-free samples.

Alpha diversity was significantly lower in the pellet samples in all four methods of measurement: observed, Chao, Shannon, and Simpson ($p<0.001$ for all) (**Figure 6**). When comparing males and females we see no significant difference in alpha diversity.

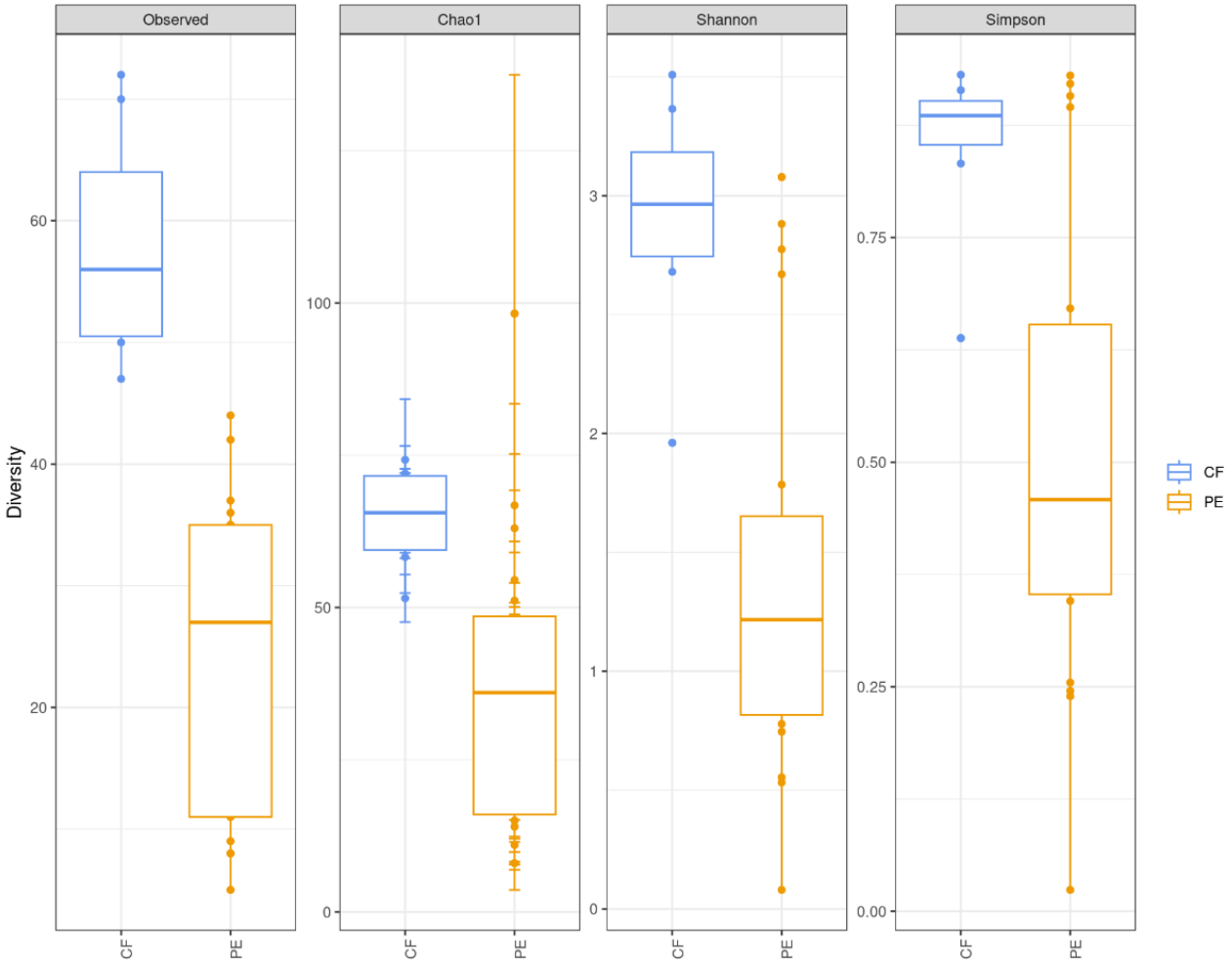

Figure 6. Alpha diversity boxplots of the cell-free and cell pellet samples measured 4 different ways. From left to right: Observed, Chao1, Shannon index and Simpson index. All 4 measures found significant differences between samples.

Beta diversity and dispersion also differed significantly between samples in both UniFrac ($p<0.001$) and Bray Curtis distances ($p<0.001$) (**Figure 7**). Sex-based differences in beta diversity were found with the female pellet samples being significantly different from both the male pellets and the cell-free samples of both sexes ($p=0.001$) (**Figures 8 and 9**).

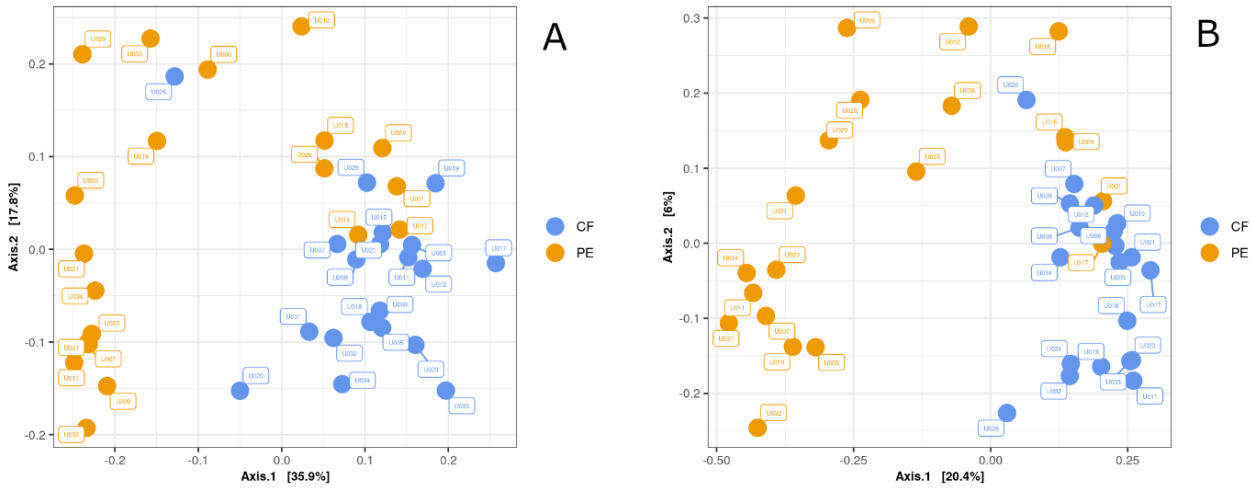

Figure 7. Principle coordinate analysis (PCoA) plots showing beta diversity of the cell-free and cell pellet samples in A) weighted UniFrac distances and B) Bray Curtis distances. Both measures found significant differences between samples.

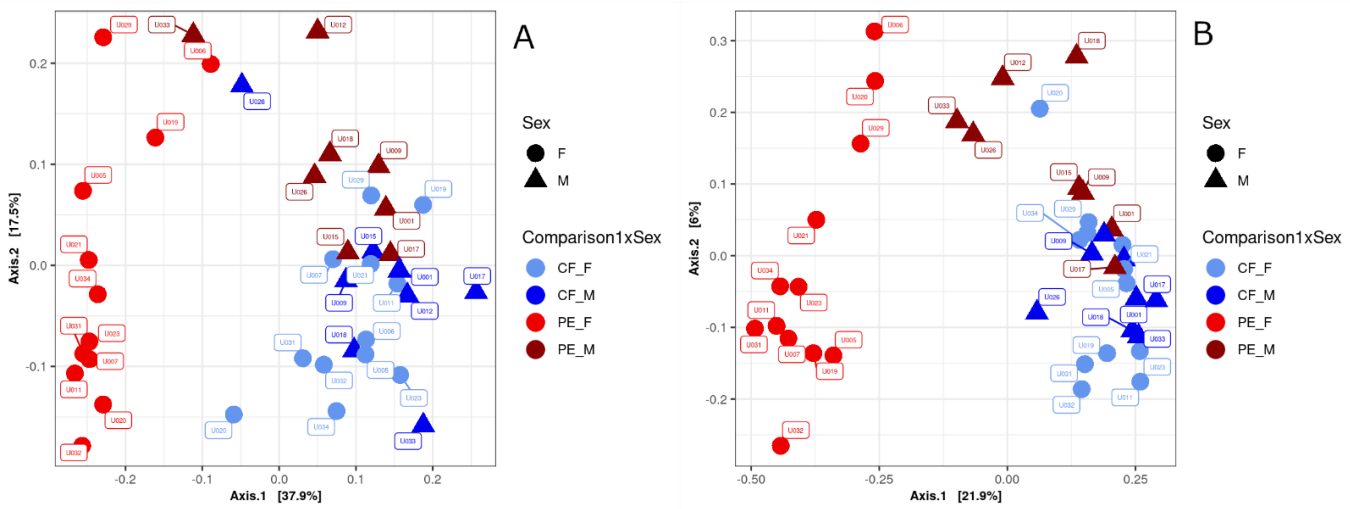

Figure 8. Principle coordinate analysis (PCoA) plots showing beta diversity of the cell-free and cell pellet samples divided by sex in A) weighted UniFrac distances and B) Bray Curtis distances. These measures found a difference between the male and female cell pellets but not cell-free samples.

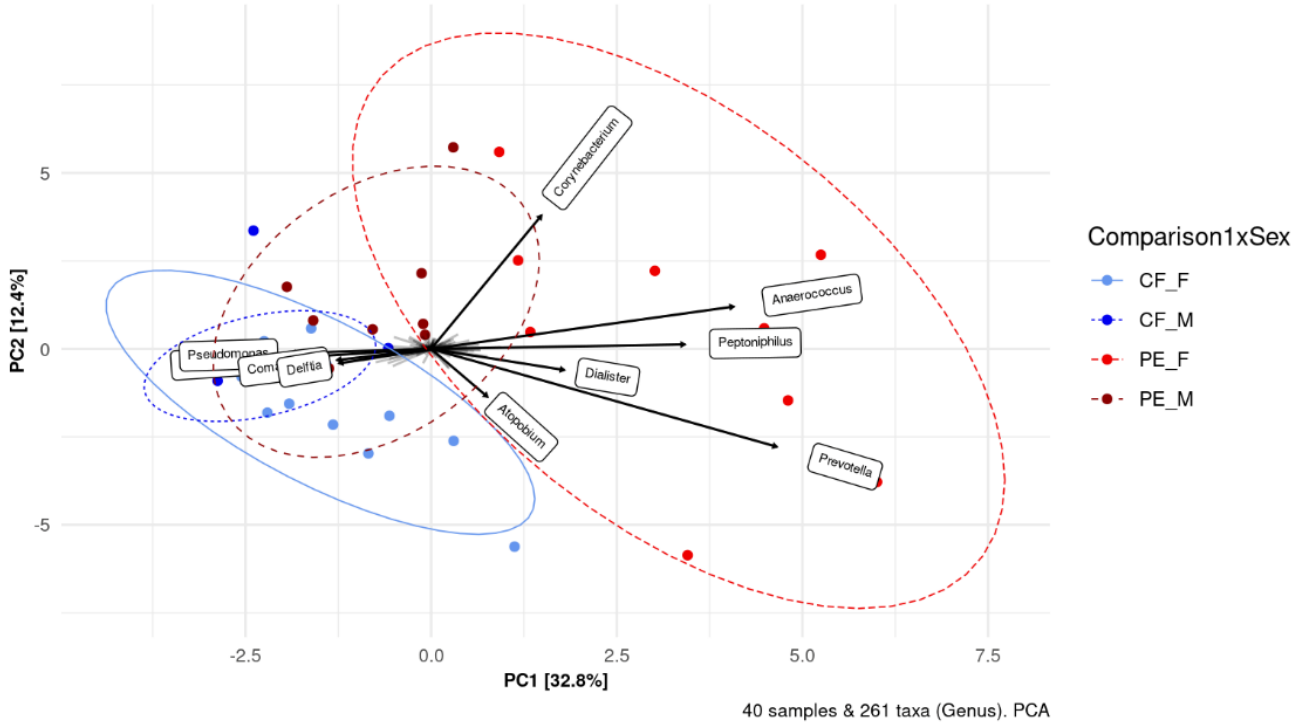

Figure 9. Principle component analysis (PCA) plot showing the beta diversity of cell-free and cell pellet samples divided by sex. The top 10 most influential taxa for determining PCA coordinates are labeled.

Differential abundance analysis shows significant differences in several taxa with cell pellets containing more: *Streptococcus, Peptoniphilus, Finegoldia, Corynebacterium, Anaerococcus* and *Staphylococcus,* while cell-free samples had more: *Acinetobacter, Delftia, Brevundimonas, Comamonas, Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium, Stenotrophomonas* and *Pseudomonas* (**Figure 10** and **Table 1**). While there were no sex-based differences in the cell-free samples, female pellet samples contained more: *Dialister, Prevotella, Finegoldia, Peptoniphilus, Anaerococcus, Fusobacterium, Varibaculum, Howardella* and *Lawsonella,* but less: *Acinetobacter, Delftia, Comamonas, Brevundimonas, Stenotrophomonas and Pseudomonas* (**Figure 11** and **Table 2**).

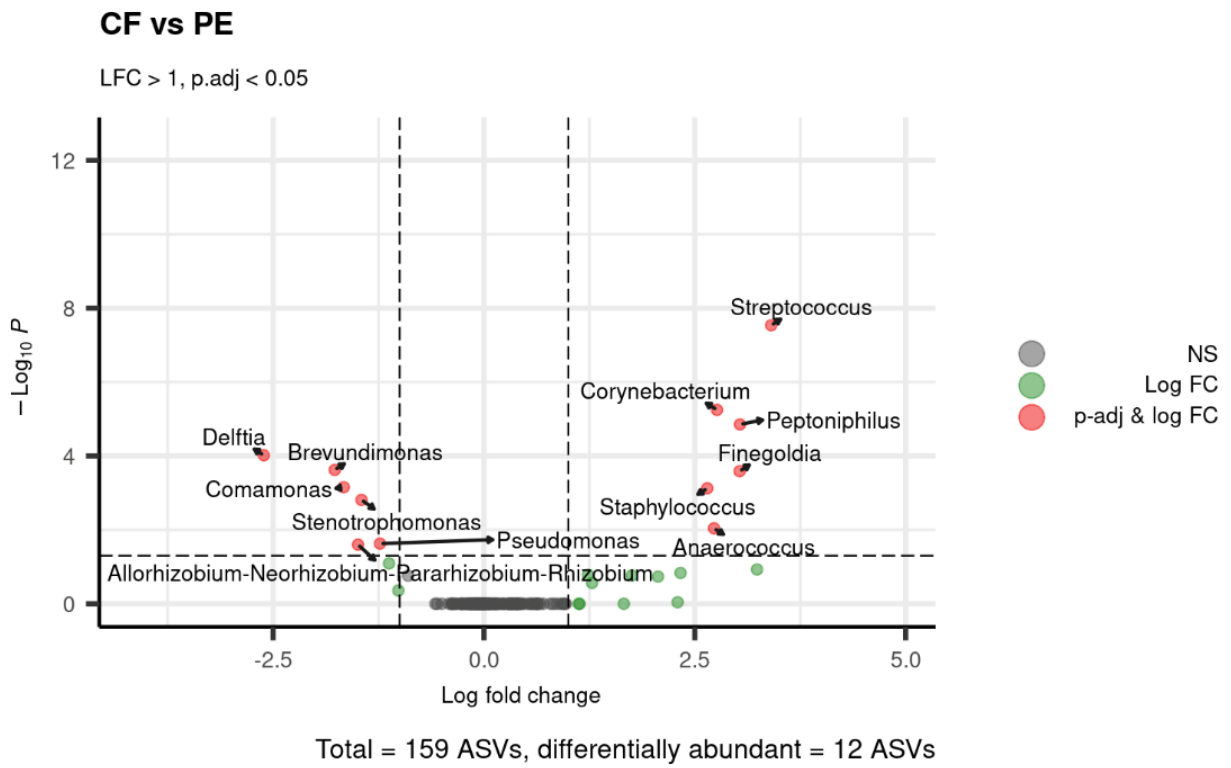

Figure 10. ANCOMBC2 volcano plot showing the differential abundance of the cell-free and cell pellet samples. Labelled taxa have significantly different abundances.

Table 1. Differential abundance of the cell-free and cell pellet samples. Log fold change (LFC) indicates how much more of a taxon was in the cell pellet (for example a value of 2 indicates that the cell pellet has 4x as much of the taxon). W statistic is a test statistic that is equal to LFC/standard error. The *P* value is obtained by using pairwise comparisons of the W statistic and the adjusted *P* value controls for the false discovery rate when performing multiple tests of hypotheses. Bolded *P* values are significant.

Taxon	Log fold change (SFC) in pellet	Standard error (SE)	W statistic	*P* value	Adjusted *P* value (q)
Streptococcus	3.41	0.53	6.38	1.83E-10	**2.90E-08**
Peptoniphilus	3.04	0.57	5.35	8.90E-08	**1.40E-05**
Finegoldia	3.03	0.63	4.79	1.68E-06	**2.58E-04**
Corynebacterium	2.76	0.50	5.51	3.57E-08	**5.64E-06**
Anaerococcus	2.73	0.68	4.01	6.04E-05	**9.07E-03**
Staphylococcus	2.65	0.58	4.57	4.92E-06	**7.48E-04**
Pseudomonas	-1.23	0.33	-3.78	1.58E-04	**2.35E-02**
Stenotrophomonas	-1.45	0.33	-4.41	1.02E-05	**1.55E-03**
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	-1.49	0.40	-3.76	1.70E-04	**2.51E-02**
Comamonas	-1.66	0.36	-4.58	4.57E-06	**6.99E-04**
Brevundimonas	-1.77	0.37	-4.81	1.54E-06	**2.38E-04**
Delftia	-2.61	0.52	-4.99	6.12E-07	**9.54E-05**

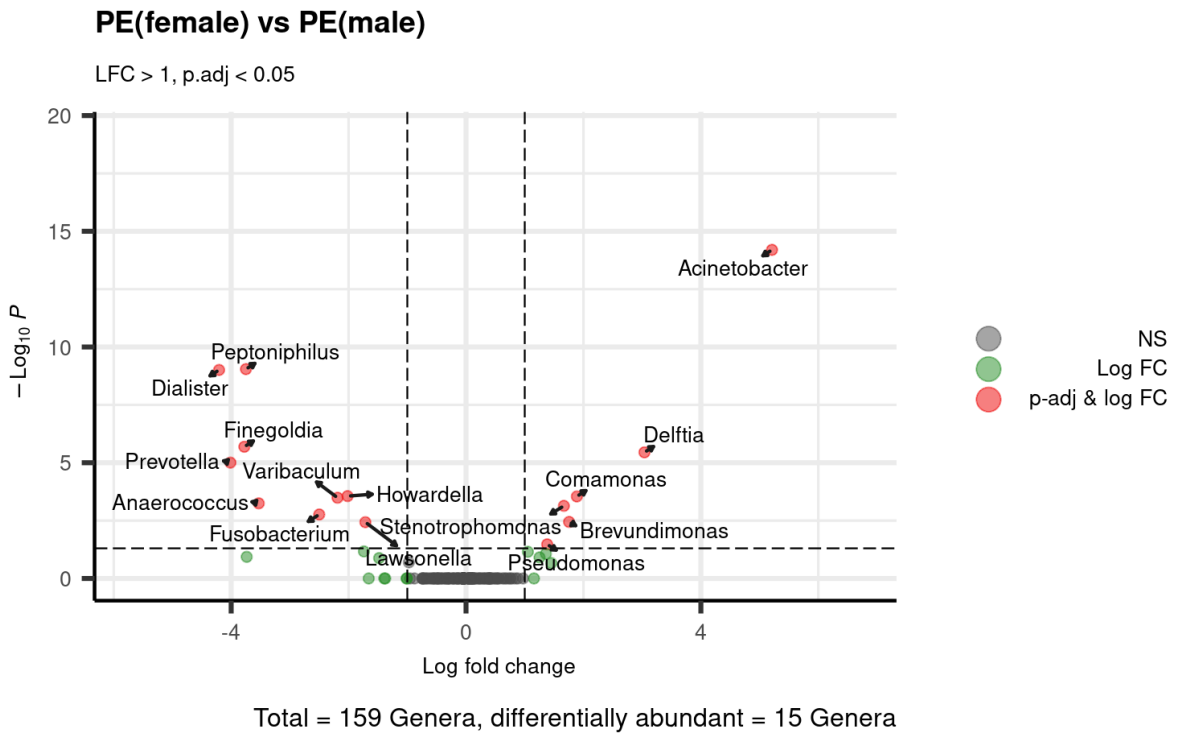

Figure 11. ANCOMBC2 volcano plot showing the differential abundances of the male and female cell pellet samples. Labelled taxa have significantly different abundances.

Table 2. Differential abundance of the male and female cell pellet samples. Log fold change (LFC) indicates how much more of a taxon was in the male cell pellet. W statistic is a test statistic that is equal to LFC/SE. The *P* value is obtained by using pairwise comparisons of the W statistic and the adjusted *P* value controls for the false discovery rate when performing multiple tests of hypotheses. Bolded *P* values are significant.

Taxon	Log fold change (LFC) male	Standard error (SE)	W statistic	*P* value	Adjusted *P* value (q)
Acinetobacter	5.21	0.63	8.23	1.83E-16	**6.36E-15**
Delftia	3.04	0.57	5.32	1.03E-07	**3.57E-06**
Comamonas	1.89	0.42	4.46	8.24E-06	**2.86E-04**
Brevundimonas	1.76	0.45	3.88	1.04E-04	**3.61E-03**
Stenotrophomonas	1.67	0.39	4.25	2.11E-05	**7.32E-04**
Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium	1.45	0.53	2.73	6.25E-03	0.22
Pseudomonas	1.38	0.42	3.30	9.69E-04	**0.03**
Schumannella	1.36	0.45	3.03	2.48E-03	0.09
Shinella	1.25	0.43	2.92	3.56E-03	0.12
Staphylococcus	1.16	0.83	1.40	0.16	1.00
Streptococcus	0.88	0.77	1.14	0.25	1.00
Corynebacterium	-0.54	0.74	-0.73	0.47	1.00
Peptostreptococcus	-1.48	0.51	-2.89	3.81E-03	0.13
Lawsonella	-1.71	0.44	-3.89	1.01E-04	**3.70E-03**
Mobiluncus	-1.74	0.56	-3.10	1.96E-03	0.07
Howardella	-2.02	0.45	-4.47	7.98E-06	**2.77E-04**
Varibaculum	-2.19	0.49	-4.43	9.40E-06	**3.26E-04**
Fusobacterium	-2.50	0.61	-4.07	4.70E-05	**1.72E-03**
Anaerococcus	-3.53	0.82	-4.31	1.64E-05	**5.69E-04**
Lactobacillus	-3.74	1.27	-2.93	3.36E-03	0.12
Peptoniphilus	-3.75	0.56	-6.67	2.60E-11	**9.04E-10**
Finegoldia	-3.78	0.70	-5.42	5.89E-08	**2.04E-06**
Prevotella	-4.01	0.78	-5.14	2.78E-07	**9.91E-06**
Dialister	-4.21	0.63	-6.66	2.67E-11	**9.81E-10**

4. Discussion

4.1 Evidence of an intracellular microbiota within urothelial cells

The story of our current understanding of urinary microbiome persistence is one of extracellular bacteria. In this, bacteria remain within the urinary system through adaptations such as type 1 fimbriae, which allow physical attachment to the urothelial cells. One commonly cited bacterium that attaches this way is *E. coli*, though this is mainly described in the context of pathogenesis.[135] An inconsistency with this line of thinking is that *E. coli* makes up a minority of the urinary microbiome and this doesn't explain the large presence of bacteria without fimbriae.

Using flow cytometry, we were able to visualize intracellular bacteria within the urothelial cells of every subject. This was not a trivial number of intracellular bacteria either, as a median of 14% of urothelial cells contained bacteria. While we were unable to count the number of bacteria within each individual cell using flow cytometry, from visual inspection we can see that many urothelial cells contain what appear to be multiple bacteria (**Supplemental Figure 2**). When we visualize the samples using SEM, we can clearly see bacterial communities aggregated together and often embedded within or on top of a biofilm-like structure (**Figure 1**). That these bacteria remain aggregated despite sonication suggests that they are the same intracellular bacteria that were seen on flow cytometry and that these biofilm-like assemblies' act in a structural role.

Most urothelial cells remain within the bladder, but when the urothelial cells were exfoliated, we detected the polymicrobial communities within. From this, we extrapolate that the non-exfoliated cells also contain intracellular bacteria. Intracellular polymicrobial communities within biofilm-like structures is a possible explanation for the persistence of the urinary microbiome within the harsh environment of the urine. While this study was not designed to confirm the presence or molecular structure of these biofilm-like structures, the imaging is quite suggestive (**Supplemental Figure 3**). Previous research has shown that intracellular biofilms consisting of polysaccharides and uroplakin allow pathogenic bacteria to persist and cause recurrent urinary tract infections, and it is likely that intracellular biofilms serve a similar role with commensal organisms.[110,111] With SEM we can look at the morphology the bacteria and clearly see both rods and cocci in multiple subjects, suggestive of polymicrobial communities (**Figure 2**), which is confirmed by the metagenomic data.

Our findings of IBCs in every subject is in contrast to previous work done by Rosen et al.[110] In 2007 the authors were unable to find IBCs in any of the asymptomatic subjects but they did find some in those with history of *E. coli* urinary tract infections. The discrepancy is due to several reasons. First, they only explored intracellular bacteria by immunofluorescence or SEM when bacterial aggregates were observed using light microscopy. Second, as they focused their research on *E. coli*, they used an anti-*E. coli* antibody. We used a more generic antibody (anti-Enterobacteriaceae common antigen), though there is reportedly some cross reactivity with the anti-*E. coli* antibody. Finally, the use of imaging flow cytometry allows for a more thorough and systematic assessment.

Overall, we find that the urinary microbiome of every individual, including males and those without previous UTIs, has a substantial portion that is intracellular within urothelial cells. This finding is significant in that it opens a new area that needs to be explored to fully understand the urinary microbiome. These intracellular bacteria within biofilm-like structures explain the urinary presence of non-pathogenic bacteria and those without fimbriae. It also offers a possible mechanism for asymptomatic bacteriuria and recurrent urinary tract infections. If we were to think of recurrent UTIs as a disease of microbiome dysbiosis, much like we do for *Clostridioides difficile* infections, then this would explain high rates of recurrence after antibiotic treatment and would also provide new targets for therapeutics development.[72] Beyond the urinary tract, our findings have possible implications for microbiome research in other organ systems, for if commensal organisms can reside within urinary endothelial cells then the same may be true in the gastrointestinal or respiratory tracts.

4.2 DNA extraction targeting intracellular biofilms defines a distinct profile of the urine microbiome.

We can draw several conclusions from our division of the urine samples into the intracellular (cell pellet) and extracellular (cell-free). First, we found that there was a strong correlation ($p<0.0001$) between the amount of bacterial DNA in the cell pellet and the cellularity of the urine (**Figure 3**). The lack of correlation with the cell-free DNA indicates that most of the urinary microbiome is contained within urothelial cells. Metagenomic analysis reveals a similar finding

with the cell-free samples having significantly lower sequencing coverage than the pellets, and therefore a higher proportion of contaminant DNA (**Figure 4**).

Outside of sequencing coverage, there are also significant differences in the relative abundances, alpha diversity, beta diversity and differential abundance when comparing the cell-free and pellet samples. Interestingly, alpha diversity was lower in the cell pellet. An explanation for this is that the cell-free sample, with its lower sequencing coverage has a higher proportion of contaminant DNA than the pellet (though this study did include a negative control to account for this). Another possibility is that we are detecting microbial cell-free DNA from the blood. Recent advances in metagenomics have found that human blood contains microbial cell-free DNA in low amounts, even in healthy patients.[136] This microbial DNA is thought to be introduced through transient disruptions in epithelial barriers and doesn't necessarily represent active infection. We also know from applications such as fetal testing that cell-free DNA is capable of being filtered through the kidneys into urine.[137] Regardless, with the lower amount of cell-free DNA, the higher alpha diversity may represent the presence of a few non-significant bacteria, rather than a more relevant, diverse polymicrobial community. Beta diversity too differed between sample types, indicating that the intracellular microbiome is distinct from its extracellular component.

Looking into the relative abundances of individual taxa we see that the cell-free samples had more *Acinetobacter* while pellets contained more *Peptoniphilus, Streptococcus, Finegoldia* and *Staphylococcus*. These variations are also visible when looking at differential abundances (**Table 1**). For example, we see that there is 11 times more *Streptococcus*, and 8 times more of both *Peptoniphilus* and *Finegoldia* in cell pellets while *Delftia* is 6 times more common in cell-free urine.

Intestinal dysbiosis of *Streptococcus* species, a diverse group of bacteria, has been implicated in several medical conditions ranging from psoriasis to atherosclerosis.[138,139] While we do not have the species level data on the *Streptococcus* in our participants' urinary microbiome, many *Streptococcus* species are known to form biofilms such as *S. mutans* on teeth and *S. pneumoniae* in the respiratory tract.[140,141] Given this, it seems reasonable to hypothesize that similar biofilm processes are occurring within the urothelial cells of the bladder. Besides *Streptococcus*, the other common intracellular bacterial species of *Finegoldia* and *Peptoniphilus* have also been

described forming biofilms[142–144]. Curiously, *Delftia*, a species seen more in cell-free samples, has also been associated with biofilm formation but conversely, it can also interfere with quorum sensing and biofilm formation.[145,146]

Important to note is the underrepresentation of certain uropathogens. Our analysis shows that there is minimal *Klebsiella* and *Proteus* while a few subjects have a significant amount of *Escherichia-Shigella* in their cell pellets (**Figure 5** – subjects 001 and 019). This is in keeping with previous research which has shown that *E. coli*, specifically UPEC, can form intracellular bacterial communities.[110–113] While we don't have the specific sequencing data to tell if the *Escherichia-Shigella* seen is UPEC or not, we know that neither subject had UTI at the time of sample collection and in fact neither subject (one male, and one female) had ever been diagnosed with a UTI. This re-affirms the idea that the presence of *Escherichia* species in urine does not always represent infection, and it also suggests that *Proteus* and *Klebsiella* species may play less of a role in the healthy urinary microbiome.

Altogether the results of this analysis indicate that the diversity of the intracellular component of the urine microbiome is quite distinct when compared to the extracellular component. We found that there is more bacterial DNA intracellularly and that specific genera of bacteria are more abundant intracellularly. This highlights the importance of the DNA extraction technique when performing any microbiome analysis. Going forward, further studies on the urinary microbiome should take this into account, lest they risk overlooking a fundamental aspect of the microbiome.

4.3 Sex-based differences of the urine microbiome

When we look at sex-based differences we find that while males and females have a similar proportion of urothelial cells containing bacteria (12.6% in females vs 15.7% in males), females overall had more intracellular bacteria (0.61 cells with IBC/μL in males vs 4.89 cells with IBC/μL in females) due to a more cellular urine (41.68 cells/μL in females vs 4.97 cells/μL in males). Although we now know that virtually every individual has bacteria in their urine at all times, previous studies found that females have a higher prevalence of asymptomatic bacteriuria (20-50% in females over 80 compared to 6-20% in males).[147]

The concept of asymptomatic bacteriuria is now more of an artifact of microbiologic diagnostics rather than the lack of actual sterile urine. Females were thought to have more asymptomatic

bacteriuria because they had more bacteria in the urine on average, which therefor could be cultured more often. Estrogen has been shown to increase urothelial cell proliferation and a possible explanation for the higher number of bacteria in the urine of females is the increased number of urothelial cells (some of which contain bacteria), rather than anatomical differences such as a shorter urethra.[148] Together this leads to a possible model for asymptomatic bacteriuria where urothelial cells containing commensal intracellular bacteria are exfoliated, exposing the bacteria to the urine where they are available for culture (**Figure 12**).

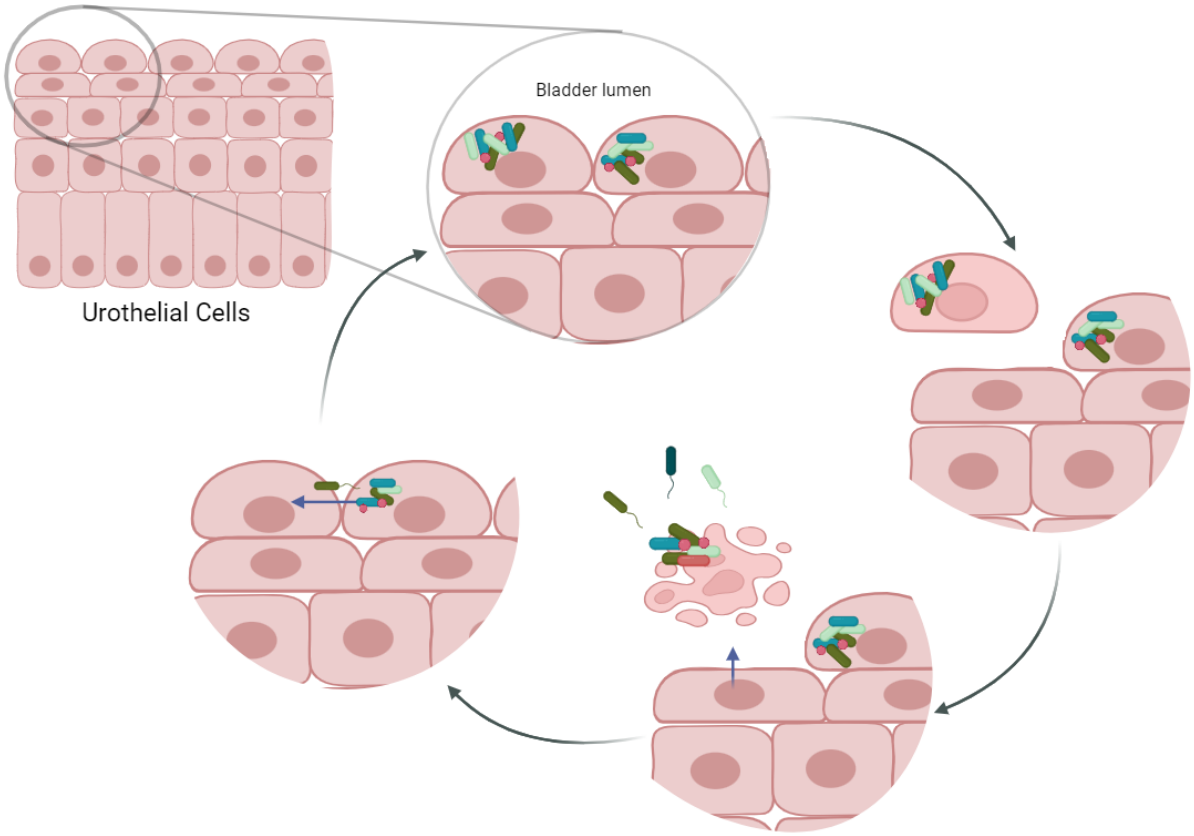

Figure 12. A proposed model of the mechanism of urinary microbiome persistence. Bacteria live in polymicrobial aggregates within urothelial cells and can propagate between cells. When the urothelial cell is exfoliated or lysed, bacteria are released into the urine.

Beta diversity and differential abundance differed significantly between the cell pellets of males and females whereas there was no difference in the cell-free samples (**Figure 11**) (**Table 2**). For example, the female pellets contained 19 times more *Dialister* and 34 times less *Acinetobacter*.

Previous studies have found sex-based differences in differential abundances and a similar study by Pohl et al that assessed the urinary microbiome in asymptomatic males and females found that beta diversity differed but the genus that differed most significantly was *Lactobacillus* (they did find more *Streptococcus* in males but not reaching significance) [46,149,150].

The lack of difference between male and female cell-free samples in our study again points towards the cell pellet samples being more of a true representation of the urinary microbiome and reinforces the idea that most of the urine microbiome is contained intracellularly. If the ascending model for UTIs/bacteriuria was the primary mechanism of bacterial acquisition, then we would expect the cell-free samples in males and females to differ in their diversity, given the anatomical differences. This is not the case, and it suggests that differing intracellular bacteria are the cause for sex-based differences, rather than anatomy. As to why females and males have different intracellular urinary microbiomes, further research is needed. Some possible explanations include antibiotic use due to previous UTIs (50% of females in this study) and hormonal differences.

By including intracellular bacteria into our understanding of the urinary microbiome, we can get a more accurate understanding of sex-based differences in the microbiome. Doing so, we can draw connections from the dysbiosis of certain taxa microbiome-associated conditions, some of which are more prevalent in females (i.e., interstitial cystitis, urgency urinary incontinence and urinary tract infections).[50,65,72]

4.4 Limitations

This study did have several limitations. Samples were collected from first-catch or midstream and not from a transurethral catheter or suprapubic aspiration. In theory this could introduce some contamination, but previous studies have found that the differences between midstream voiding and sterile catheterization are minimal.[150,151] In fact, data from this study shows that the amount of DNA introduced from the urethra (which would have been part of the cell-free sample) is minimal compared to the DNA contained within urothelial cells.

Another minor aspect is the heterogeneity between the timing of urine samples - some were first void, while others were collected later in the day. In urine there can be some small variations

between the microbiome of first-catch and midstream urine, but there is no evidence of diurnal microbiome changes such as in the intestinal microbiome.[152,153]

SEM was used as an exploratory/screening method to identify bacterial communities. Without using an automated detection system, we were bound to miss some examples of bacteria and polymicrobial communities. This is likely why we did not visualize polymicrobial communities in 100% of participants despite the 16S sequencing indicating otherwise. In addition, we used an anti-ECA antibody which can detect many gram-negative bacteria, but gram-positive bacteria such as *Lactobacillus* and *Streptococcus* sp. would have been underrepresented in flow cytometry as well as the SEM images. This underrepresentation could explain why cocci, which are more likely to be gram-positive, were not seen in the SEM of all participants.

The study was not designed to determine the composition of the biofilm-like structures and therefore we cannot definitively call them biofilms. Further research will be needed in this area. In addition, while imaging flow cytometry can confirm the presence of intracellular bacteria, it cannot tell us how many bacteria are within each cell.

5. Conclusion

We recruited 20 participants with a variety of ages and diverse backgrounds who had not had a UTI in the previous 6 months. Overall, we found evidence of an intracellular component of the urinary microbiome within every participant. Commensal intracellular bacteria were found together in polymicrobial communities, often embedded within a biofilm-like structure. This was not a rare occurrence, as 14% of urothelial cells were found to contain bacteria and the amount of bacterial DNA correlated only with the intracellular component, and not the extracellular. There were significant differences in alpha diversity, beta diversity, relative abundance, and differential abundance when the extracellular and intracellular urine components were compared. A large intracellular component could explain how the urine microbiome is able to persist within the human urinary tract which flushes itself multiple times a day.

Sex-based differences were also identified, with females having a higher number of shed urothelial cells containing bacteria (though a similar proportion), and it is possible that this could explain differences in the prevalence of asymptomatic bacteriuria. Beta diversity and differential

abundance differed significantly between the intracellular components of males and females. Future studies could use these differences to explain sex-based differences in prevalences of various urologic conditions.

This is the first-time intracellular bacteria have been described in the urine of healthy individuals, representing a breakthrough in our understanding of the urinary microbiome. In addition, an important takeaway from this study was that the method of DNA extraction in microbiome analysis plays a massive role in any potential findings. Future studies on the human microbiome in any location should consider evaluating for intracellular bacteria.

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7. Appendix

Study ID#:_____

Urine Microbiome Study Questionnaire (IBC-5)

Demographics:

Date of Birth (dd- mmm-yyyy): _____/_____/_____

Sex at birth: ☐ Male ☐ Female Sex Now: ☐ Male ☐ Female

Gender/currently self identify as: ☐ Man ☐ Woman ☐ Non-binary, genderqueer, agender or a similar identity ☐ Two-spirit ☐ Prefer to self describe ☐ Prefer not to answer

Race/ethnicity:

Are you of Hispanic, Latino or Spanish origin?
☐ **No** ☐ **Yes** - please select below

☐ Mexican, Mexican American, Chicano
☐ Puerto Rican
☐ Cuban
☐ Other (Please specify)_____

How would you best describe yourself?
☐ White
☐ Black, African American
☐ American Indian or Alaska native (Please specify tribe)_____
☐ Asian Indian
☐ Japanese
☐ Native Hawaiian
☐ Chinese
☐ Korean
☐ Guamanian or Chamorro
☐ Filipino
☐ Vietnamese
☐ Samoan
☐ Other Asian (Please specify) _____
☐ Other Pacific Islander (Please specify) _____
☐ Other race (Please specify) _____

Screening Questionnaire:

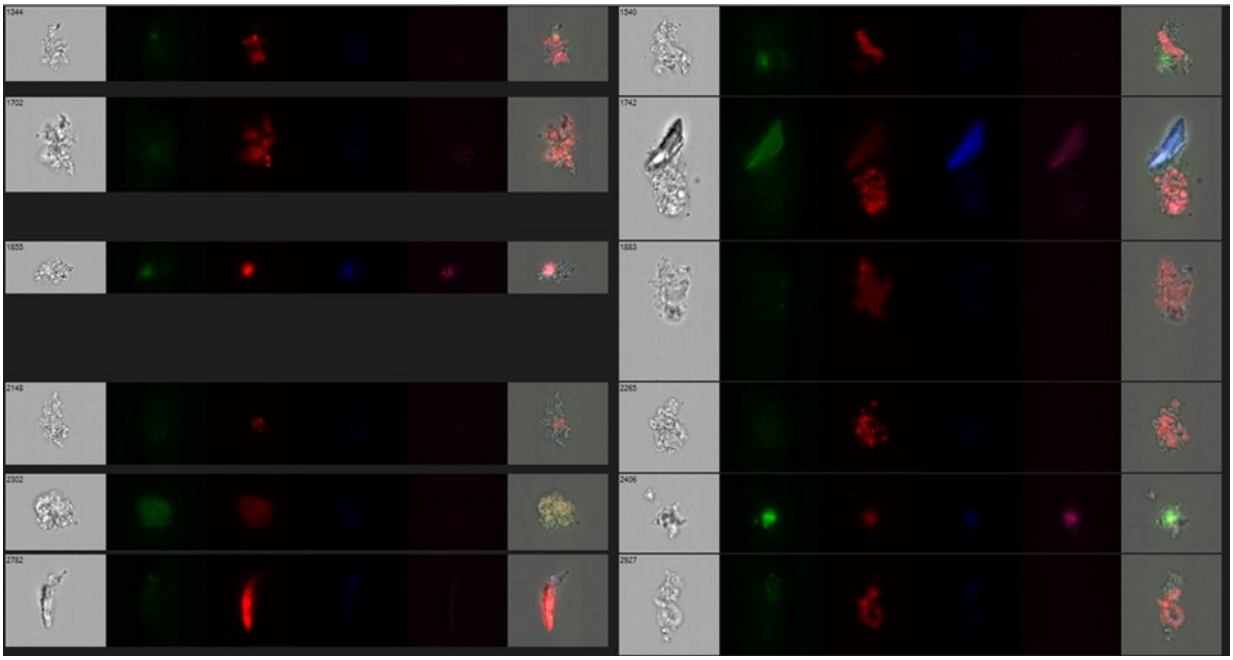
1) Have you ever been diagnosed with urinary tract infection?
☐ **No** ☐ **Yes**
If yes, have you been diagnosed with, or taken antibiotics for a urinary tract infection (UTI) within the past 6 months?
☐ **No** ☐ **Yes**

2) Are you taking any antibiotics (either for treatment of an infection or prophylaxis) currently?
☐ **No** ☐ **Yes**

3) At what age did you have your first period? (if applicable): _____

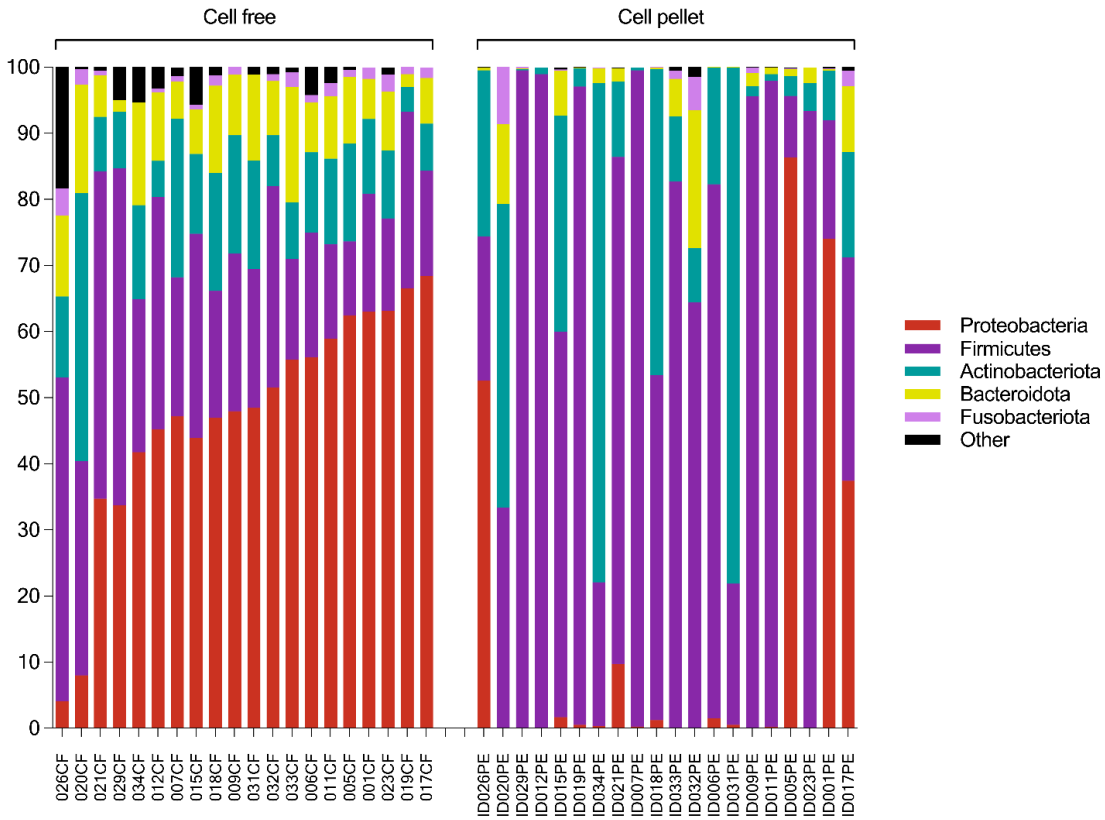
4) At what age did you enter menopause (last period)? (if applicable): _____

Supplemental Figure 1. The questionnaire given to each patient upon enrolment into the study. Subjects currently on antibiotics or who had taken antibiotics for a UTI in the past 6 months were excluded from the study.



Supplemental Figure 2. Example of imaging flow cytometry images from a participant (ID001) demonstrating bacteria within urothelial cells. Green: uroplakin III; Red: Enterobacteriaceae common antigen (bacteria); Blue: nuclear stain

Supplemental Figure 3. SEM image showing remnants of aggregated bacteria inside a biofilm-like capsule.



Supplemental Figure 4. Relative abundances of phyla of the cell-free and cell pellet samples. Subjects are sorted according to likeness of the cell-free samples.