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The urogenital microbiota in urinary tract diseases

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The urogenital microbiota in urinary tract diseases

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Editorial: The urogenital microbiota in urinary tract diseases

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Editorial on the Research Topic
The urogenital microbiota in urinary tract diseases

The urogenital microbiota (urinary, periurethral, vaginal and penile) has the potential to modulate susceptibility or severity of infections caused by recognized uropathogens (e.g. UTI, urethritis) or other urologic conditions not previously regarded as having a microbial origin (e.g. IC/BPS, interstitial cystitis, bladder cancer). In this Research Topic, a collection of studies used cutting-edge tools to analyze, or re-analyze, urogenital microbiome data from a variety of patient groups to examine associations with urinary tract disorders or between urogenital niches. Other studies developed new methods for analyzing urobiome data or for examining the effect of urogenital bacteria on the mucosa. Finally, a group of studies developed new *in vitro* and *in vivo* model systems to directly examine how specific members of the urogenital microbiome affect recognized uropathogens.

The bioinformatic tools and pipelines used to generate and analyze microbiome data are constantly advancing. New insights can be generated from re-analysis of existing samples or datasets. Joyce et al. re-analyzed the urobiome in >1,000 adult women with a wide range of urinary tract syndromes: asymptomatic, urgency urinary incontinence (UUI), stress urinary incontinence (SUI), urinary tract infection (UTI) and interstitial cystitis/painful bladder syndrome (IC/PBS). Siddiqui et al. re-analyzed 16S rRNA gene sequencing data from >200 individuals with or without mixed urinary incontinence (MUI). The new analytical approaches used in these studies revealed previously unrecognized features of the urobiome. Another consideration in microbiome research is the choice of which 16S hypervariable region to analyze. Heidrich et al. report that the V1V2 regions best capture the taxa present in male urine samples. Together, these studies highlight the importance of revisiting microbiome data as computational tools progress and of empirically determining the best experimental approach for analyzing the

urobiome of specific populations, which may be different that what it optimal for other human biological samples such as stool.

A caveat to standard 16S sequence-based microbiome data is that it cannot distinguish live bacteria from dead. Bacterial viability can be demonstrated using extended quantitative urine culture (EQUC), but this method adds a cost and labor burden and does not capture all urobiome species. The DNA-binding dye propidium monoazide (PMA), which can penetrate dead/dying cells and thus prevent PCR amplification, has been used in other microbiome studies to distinguish viable from non-viable bacteria. Lee et al. developed methods for utilizing the PMA-binding PCR assay in urine. With further refinement, PMA-based urine PCR has the potential clinical advantages of more rapid UTI diagnosis and broader organism detection and could identify situations where recalcitrant symptoms following UTI treatment are due to persistence of viable but nonculturable bacteria.

Most of the bacterial genera reported in urobiome studies had previously been recognized as vaginal bacteria. This observation, coupled with the proximity of the urethra to the vaginal introitus, suggests that microbial overlap exists between the urinary tract and the vagina in individual women. Nardos et al. performed microbial network analysis on paired vaginal and catheterized urine samples from women with and without UUI. Echoing previous studies, there was substantial overlap between vaginal and urine samples across all participants. However, the number of shared bacterial genera between the two niches was higher in UUI patients and the most abundantly shared was *Gardnerella*, while controls had lower numbers of shared bacterial genera and the most abundantly shared was *Lactobacillus*. These data suggest that women with UUI could have more frequent bladder exposures to vaginal bacteria or that they have a bladder environment more permissive to persistence of certain vaginal bacteria. Longitudinal studies in women and direct experimental investigations (like those described below) are needed to distinguish these possibilities.

The next step after discovering the existence of viable bacteria in urine samples is determining what the bacteria are doing in the bladder. Answering this question requires new *in vitro* and *in vivo* model systems. Nguyen et al. developed an *in vitro* assay to examine a biologically relevant effect that urogenital bacteria could be having on the bladder mucosa: degradation of the protective glycosaminoglycan GAG layer. This rapid, inexpensive and quantitative assay demonstrated GAG degradation by the uropathogen *Proteus mirabilis* but not by uropathogenic *Escherichia coli* (UPEC) or any of several species of urinary lactobacilli. Johnson et al. examined the ability of urinary *Lactobacillus* isolates to inhibit Gram-negative and Gram-positive uropathogenic model strains and clinical and multi-drug resistant isolates *in vitro*. There was substantial variety in the ability and mode of inhibition among *Lactobacillus* species and strains.

Previous studies developed the first *in vivo* models examining the effect of the frequent and abundant urogenital microbiome member *Gardnerella* on the bladder in mice.

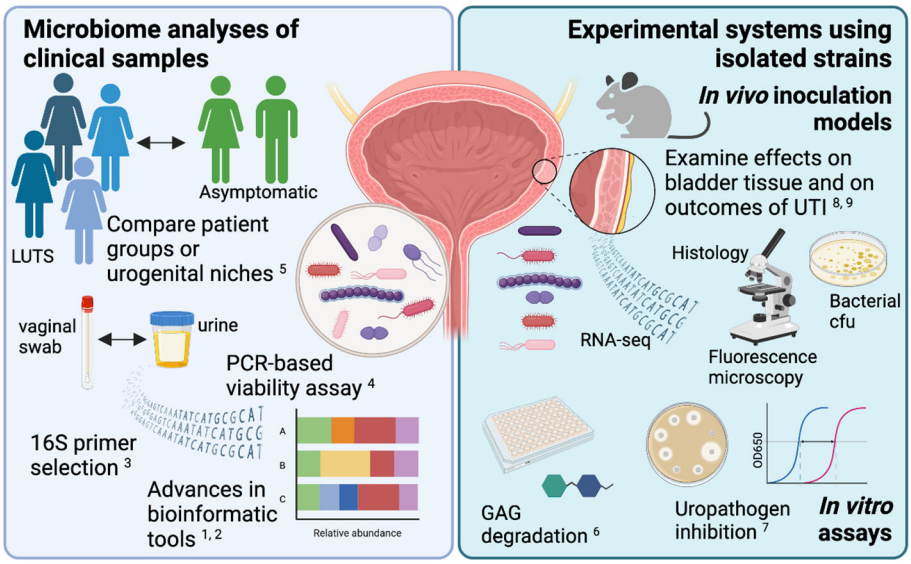

FIGURE 1
New bioinformatic tools and experimental model systems are refining our understanding of how the urogenital microbiota contributes to urinary tract diseases. The figure was created using BioRender.com.

Gardnerella is cleared from the mouse bladder within 12 hours after transurethral inoculation, but nonetheless caused urothelial exfoliation and triggered recurrent (r)UTI from quiescent intracellular reservoirs (QIRs) of UPEC. In two follow-up studies using this model (O'Brien et al. and Gilbert et al.), RNA-seq analysis revealed that bladders exposed to *Gardnerella* displayed a transcriptional signature of inflammation and urothelial turnover, both in naive mice and those harboring UPEC QIRs. The orphan nuclear receptor Nur77 was induced by *Gardnerella* in mice with QIRs, and additional studies with knockout mice demonstrated that it is required for *Gardnerella* exposure to induce UPEC rUTI (O'Brien et al.). These results established the utility of an RNA-seq approach to identify genes that mediate the effect of urogenital bacteria on UTI susceptibility. In naive mice, bladder exposure to *Gardnerella* paved the way for a subsequent UTI, lowering the dose of UPEC necessary to result in persistent infection (Gilbert et al.).

The articles in this Research Topic highlight the importance of a multi-pronged approach for urogenital microbiome research (Figure 1). The field will continue to thrive as it applies cutting-edge technologies and analytical tools to urobiome analysis in order to reveal associations between certain organisms and particular clinical phenotypes, and then uses that information to develop novel experimental model systems to directly address the mechanistic nature of these relationships. In turn, observations made in these model systems will inform future, more targeted clinical investigations of links between the urogenital microbiome and urinary tract diseases. This iterative approach has the greatest promise to yield actionable steps to improve bladder health.

Author contributions

NG wrote the initial manuscript draft and all authors edited and approved of the final editorial. NG created the figure. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Bladder Exposure to *Gardnerella* Activates Host Pathways Necessary for *Escherichia coli* Recurrent UTI

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Recurrent urinary tract infections (rUTI) are a costly clinical problem affecting millions of women worldwide each year. The majority of rUTI cases are caused by uropathogenic *Escherichia coli* (UPEC). Data from humans and mouse models indicate that some instances of rUTI are caused by UPEC emerging from latent reservoirs in the bladder. Women with vaginal dysbiosis, typically characterized by high levels of *Gardnerella* and other anaerobes, are at increased risk of UTI. Multiple studies have detected *Gardnerella* in urine collected by transurethral catheterization (to limit vaginal contamination), suggesting that some women experience routine urinary tract exposures. We recently reported that inoculation of *Gardnerella* into the bladder triggers rUTI from UPEC bladder reservoirs in a mouse model. Here we performed whole bladder RNA-seq to identify host pathways involved in *Gardnerella*-induced rUTI. We identified a variety host pathways differentially expressed in whole bladders following *Gardnerella* exposure, such as pathways involved in inflammation/immunity and epithelial turnover. At the gene level, we identified upregulation of Immediate Early (IE) genes, which are induced in various cell types shortly following stimuli like infection and inflammation. One such upregulated IE gene was the orphan nuclear receptor *Nur77* (aka *Nr4a1*). Pilot experiments in *Nur77*$^{-/-}$ mice suggest that Nur77 is necessary for *Gardnerella* exposure to trigger rUTI from UPEC reservoirs. These findings demonstrate that bladder gene expression can be impacted by short-lived exposures to urogenital bacteria and warrant future examination of responses in distinct cell types, such as with single cell transcriptomic technologies. The biological validation studies in *Nur77*$^{-/-}$ mice lay the groundwork for future studies investigating Nur77 and the Immediate Early response in rUTI.

Keywords: urinary tract infection (UTI), bacterial vaginosis (BV), Nur77, immediate early gene expression, bladder, urinary microbiome, orphan nuclear receptor 4A1 (NR4A1), RNA-seq - RNA sequencing

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INTRODUCTION

In humans, the urinary tract is the second most common site of infection, most frequently by uropathogenic *Eschericia coli* (UPEC) (Foxman, 2014). Approximately 1% of bladder infections (cystitis) progress to more serious kidney infections (pyelonephritis), and some of these become life-threatening systemic infections (Ki et al., 2004; Jolley et al., 2012). Recurrent urinary tract infections

(rUTIs) are very common: 24% of women with an initial UTI will have rUTI within 6 months, and up to 70% will have rUTI within 1 year (Foxman et al., 2000; Foxman, 2014). Approximately 1% of all women (~35 million worldwide) have six or more rUTIs each year (Foxman et al., 2000; Foxman et al., 2002; Foxman, 2014). These women are often given prophylactic antibiotics, contributing to the problem of antibiotic resistance.

It is now appreciated that UPEC has an intracellular niche within the bladder. Multiple studies of UTI and rUTI in adults and children have identified intracellular UPEC, both in bladder epithelial cells shed in urine and in bladder biopsies (Elliott et al., 1985; Rosen et al., 2007; Robino et al., 2014; Liu et al., 2016; De Nisco et al., 2019). One study using confocal microscopy detected intracellular bacteria in 36.8% of samples from children with acute UTI. Notably, a medical chart review demonstrated that the children with intracellular bacteria were significantly more likely to have a history of rUTI (OR, 8.0; 95% CI, 2.3-27.4) (Robino et al., 2014). In C57BL/6 mice, UPEC can persist within bladder epithelial cells for months after inoculation, without bacteriuria (bacteria in urine) and despite antibiotic treatment (Mulvey et al., 2001; Schilling et al., 2002; Eto et al., 2006; Mysorekar and Hultgren, 2006). In patients, most rUTIs (as many as 82%) are caused by a UPEC strain identical to that of the previous infection (Ejrnaes et al., 2006; Czaja et al., 2009; Luo et al., 2012; Skjot-Rasmussen et al., 2013; Koljalg et al., 2014), even when appropriate antibiotic therapy is given. This could reflect reintroduction of UPEC to the bladder from the gut or vaginal reservoir, but is also consistent with the concept that rUTI can be caused by emergence of the initially infecting UPEC strain from an intracellular bladder reservoir (Mulvey et al., 2001; Hickling and Nitti, 2013; Glover et al., 2014). The potential for bladder reservoirs to seed rUTI has prompted preclinical investigations into strategies to eliminate bladder reservoirs as a means of rUTI. *Gardnerella* strain JCP8151B causes epithelial exfoliation in the vagina in a mouse model (Gilbert et al., 2013). We hypothesized that *Gardnerella* could likewise cause bladder epithelial (urothelial) exfoliation and thus be a trigger of UPEC rUTI from bladder reservoirs in women. This hypothesis was further supported by three clinical observations potentially linking *Gardnerella* with UTI. First, women with bacterial vaginosis, a vaginal dysbiosis in which the vagina is overpopulated by a polymicrobial mixture of bacteria including *Gardnerella*, have an up to 13-fold higher likelihood of UTI than those with *Lactobacillus*-dominated vaginal microbiotas (OR 2.21-13.75) (Harmanli et al., 2000; Hillebrand et al., 2002; Sharami et al., 2007; Sumati and Saritha, 2009; Amatya et al., 2013). Second, women with rUTI who received vaginal interventions that influence the vaginal microbiota (e.g., vaginal estrogen, probiotic intravaginal *Lactobacillus crispatus*) experienced fewer rUTIs than those who received placebo (Stapleton et al., 2011; Rahn et al., 2014; Sadahira et al., 2021). Third, multiple urinary microbiome studies have detected *Gardnerella* in urine collected directly from the bladder (*via* suprapubic aspiration or catheterization), suggesting that *Gardnerella* routinely gains access to the bladder in some women (Wolfe et al., 2012; Pearce et al., 2014; Pearce et al., 2015; Thomas-White et al., 2016; Gottschick et al., 2017). Intriguingly, *Gardnerella* can co-aggregate with UPEC in an *in vitro* biofilm assay (Castro et al., 2016), suggesting possible synergy between these bacterial species.

To test our hypothesis, we developed a mouse model to determine whether *Gardnerella* could cause exfoliation in the bladder and UPEC emergence from bladder reservoirs (Gilbert et al., 2017; O'Brien et al., 2020). We found that in mice with intracellular UPEC reservoirs in the bladder, two transurethral *Gardnerella* inoculations triggered UPEC emergence from reservoirs, leading to rUTI. *Gardnerella* exposure caused urothelial apoptosis and exfoliation, which likely enabled UPEC to emerge from reservoirs. Notably, *Gardnerella* did not stably colonize the bladder, demonstrating that a brief exposure was sufficient to elicit UTI pathogenesis. Here we sought to further characterize the effect of *Gardnerella* bladder exposures in order to identify host processes involved in UPEC rUTI that could serve as potential biomarkers or therapeutic targets. We performed RNA sequencing (RNA-seq) on bladders harboring intracellular UPEC reservoirs, with and without exposure to *Gardnerella*. We found that host gene expression changes following *Gardnerella* exposure were modest, in keeping with a bacterial exposure that does not result in stable colonization. However, gene set enrichment analyses revealed upregulation of many host pathways. Some of the pathways, such as apoptosis and inflammatory cytokines, corroborated the phenotypic results we previously reported in this model, and other pathways were generally related to UTI. Five genes in the Immediate Early response pathway were significantly upregulated, including *Nur77* (aka *Nr4a1*). Notably, Nur77$^{-/-}$ mice were protected from *Gardnerella*-induced rUTI, confirming its role in this model.

RESULTS

Mouse Model of Gardnerella Bladder Exposure

We performed RNA-seq on whole bladders to identify early host responses to *Gardnerella* bladder exposure that could contribute to UPEC rUTI. **Figure 1** outlines the experimental timeline. First, we established UPEC bladder reservoirs by administering an initial intravesical inoculation (directly into the bladder through the urethra) of 10^7 colony-forming units (CFU) of the UPEC strain UTI89. Mice that cleared UPEC bacteriuria by 4 weeks post infection (wpi), as determined by weekly urine dilution plating and CFU enumeration, were inoculated intravesically with *Gardnerella* strain JCP8151B or vehicle (PBS) as a control. Our previous experiments demonstrated that *Gardnerella* is cleared by 12 hours (h), so we refer to this as an 'exposure' rather than an infection. One group of mice received a single exposure and bladders were collected 12 h later in order to examine early host responses to *Gardnerella*. These groups are labelled PBS-1 and *Gard*-1. Our previous studies demonstrated that two exposures to *Gardnerella* were required to cause urothelial exfoliation and to trigger UPEC rUTI. Therefore,

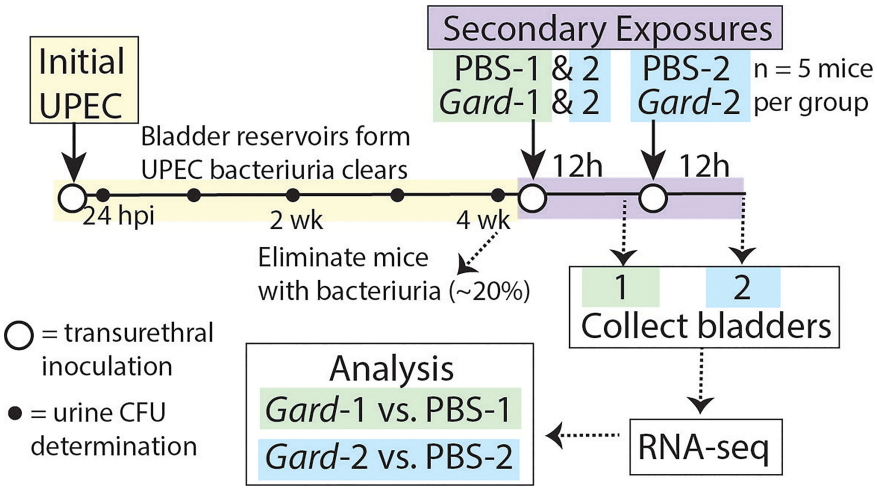

FIGURE 1 | Mouse model schematic for RNA-seq experiments. The time line of the model depicts the 'Initial UPEC' reservoir formation phase in yellow and the 'Secondary Exposure' phase in purple. Female C57BL/6 mice were inoculated transurethrally (open circles) with UPEC and bacteriuria was monitored weekly (closed circles). Mice that cleared UPEC bacteriuria were inoculated transurethrally with either PBS as a control or *Gardnerella*. Twelve hours later Exposure Group 1 was sacrificed while Exposure Group 2 received an additional transurethral inoculation and was sacrificed twelve hours (h) later. For the bladder titer experiments described in **Figures 4**, **5** the second exposure occurred one week later instead of 12 h later and bladders were collected 72 h after the second exposure.

to understand the host responses to *Gardnerella* that could ultimately lead to exfoliation and rUTI, another group of mice, labelled PBS-2 and *Gard*-2, received two exposures, 12 h apart, and bladders were collected 12 h after the second exposure (Gilbert et al., 2017; O'Brien et al., 2020). Consistent with our previous report that UPEC emergence from reservoirs usually occurs 24-72 h after *Gardnerella* exposure, only one mouse in the current study (in the *Gard*-1 group) had detectable reservoir emergence indicated by UPEC titers in urine collected immediately prior to sacrifice.

RNA was extracted from bladders from each experimental group (5 mice per group) and used for RNA-seq to assess host gene expression changes as a result of *Gardnerella* exposures. A total of 640,624,040 RNA-seq reads were generated. Of these, 457,166,961 could be aligned to the *Mus musculus* reference genome. Further details of the RNA-seq reads are found in **Supplementary Table 1**. Principal component analysis and multi-dimensional scatter plots did not reveal obvious clustering of any of the individual exposure groups (not shown).

Gene Set Enrichment Analysis Shows Pathways Related to Urothelial Turnover and Inflammation

We determined which gene ontology (GO) terms were differentially expressed following each *Gardnerella* exposure relative to PBS controls from the same time points (i.e. PBS-1 *vs. Gard*-1 and PBS-2 *vs. Gard*-2). Four GO molecular functions and 54 GO biological processes were significantly down-regulated (FDR adjusted $P < 0.05$, $\log_2FC > 2$) following the first *Gardnerella* exposure (**Figure 2A**). The majority of the affected pathways were related to host immune and inflammatory processes (**Supplementary Tables 2, 3**). More substantial differences were seen following the second *Gardnerella* exposure (**Figure 2A**): 215 GO molecular functions and 1,488 GO biological processes were significantly up-regulated (FDR adjusted $P < 0.05$, $\log_2FC > 2$). In addition to immune and inflammatory processes, many GO functions were related to urothelial integrity and turnover (**Supplementary Tables 4, 5**). Also, 33 GO terms related to apoptosis were increased, which is consistent with our previous observation of increased cleaved Casp-3 staining and TUNEL-positive urothelial cells following two *Gardnerella* exposures (Gilbert et al., 2017).

KEGG Pathway Analysis Shows a Dynamic Response to Gardnerella Exposure

We performed a similar gene set analysis (comparing PBS-1 *vs. Gard*-1 and PBS-2 *vs. Gard*-2) using the KEGG Pathway database. Echoing the results from the GO term analysis, more substantial changes occurred following two *Gardnerella* exposures. Compared to one *Gardnerella* exposure, two exposures resulted in a greater number of pathways affected and greater with greater fold changes (**Figures 2B, C** and **Table 1**). Our previous studies showed that two exposures are necessary to elicit significant urothelial exfoliation and rUTI, which is consistent with greater changes to gene expression in the bladder after the second *Gardnerella* exposure.

The pathways that were activated following one *Gardnerella* exposure were primarily related to metabolism, while the pathways with decreased expression included several related to the immune system and signaling (**Supplementary Table 6**). In contrast to one *Gardnerella* exposure, where more pathways were downregulated than upregulated, after two *Gardnerella* exposures all but two of the affected pathways were upregulated (**Supplementary Table 7**). Consistent with our previous observation of urothelial exfoliation at this time point,

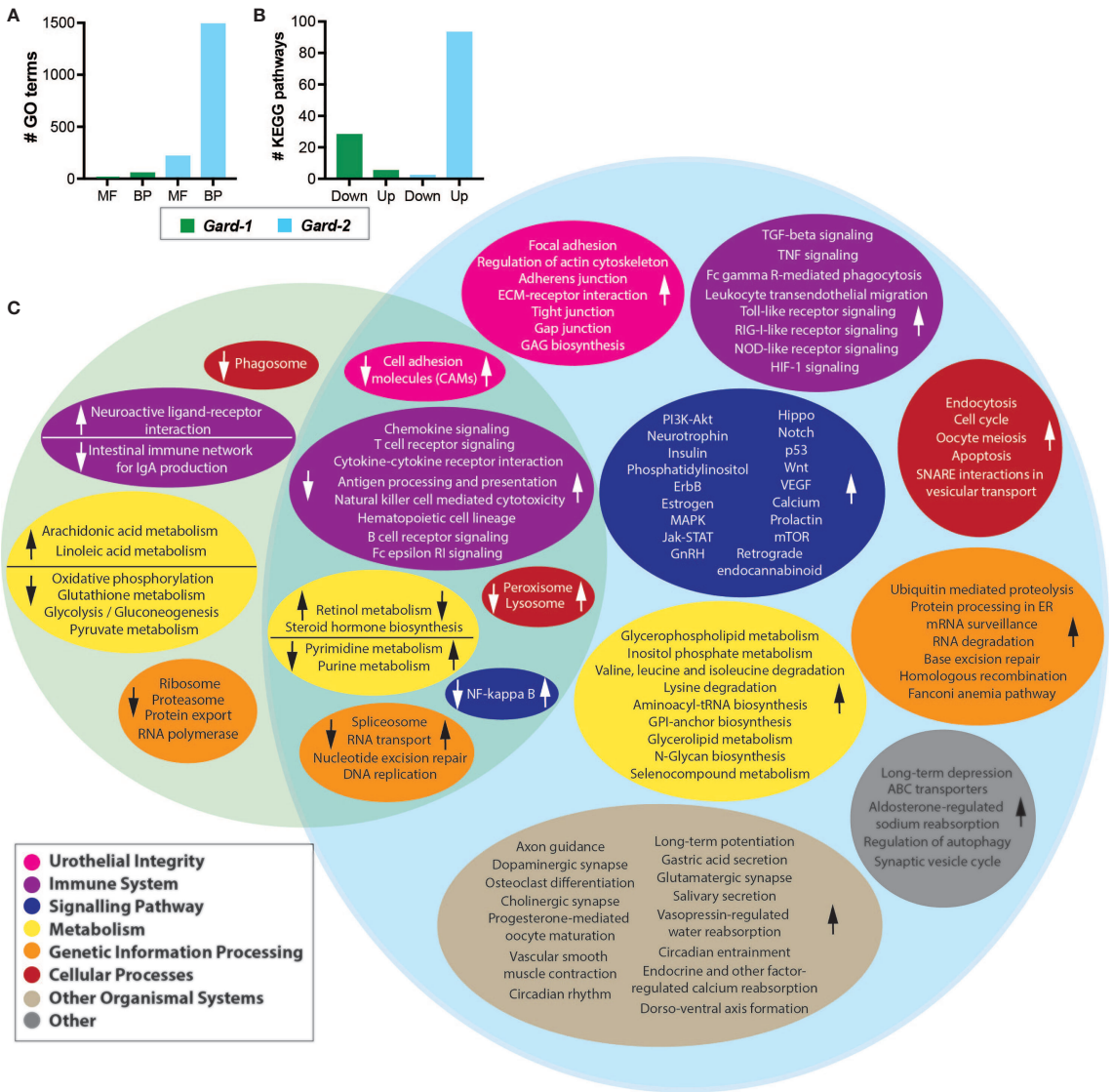

FIGURE 2 | Differential expression analysis. Shown are the host gene expression pathways that were differentially expressed following each *Gardnerella* (Gard) exposure. **(A)** The indicates number of Gene Ontology (GO) terms were significantly up- or down-regulated. MF, molecular functions; BP, biological processes. **(B)** The indicated number of KEGG pathways were significantly up- or down-regulated following *Gardnerella* exposure. **(C)** The Venn diagram displays KEGG pathways that were significantly different between PBS-1 and *Gard*-1 (left, green circle) or PBS-2 and *Gard*-2 (right, blue circle). Pathways with similar functions are organized into color-coded ovals as indicated in the figure legend. The direction of the change in the *Gard* group relative to the PBS group is indicated by arrows in each colored oval. The overlapping section of the Venn diagram indicates pathways impacted in both comparisons, with the arrows on the left in each colored oval indicating the direction of the change for *Gard*-1 and the arrow on the right indicating the direction of the change for *Gard*-2.

several affected pathways were related to epithelial integrity and renewal, such as focal adhesion, cell junctions (adherens, tight, gap), actin cytoskeleton regulation, and Wnt signaling. Many of the upregulated pathways were related to mucosal immune responses, as would be expected following a bacterial exposure. In addition to pathways specific for certain immune cell types, such as T cells, NK cells, and B cells, changes were observed for immune processes like phagocytosis, antigen processing and presentation and cytokine/chemokine signaling. Several signaling pathways related to inflammation and immunity were also affected, including PI3K-Akt, NF-κB, Jak-STAT and Hippo.

When considering the temporal dynamics of the pathway changes, we found that 20 pathways were significantly different in expression at both time points, but for each of these the change was in opposite directions (**Figure 2C** arrows; **Table 2**). For example, pathways relating to retinol metabolism and steroid hormone biosynthesis were upregulated after the first exposure but downregulated after the second. In contrast, inflammatory pathways like cytokine-cytokine receptor interaction, chemokine

TABLE 1 | Host Pathways Most Affected by *Gardnerella* Bladder Exposures.

	KEGG Pathway	logFC	P-value (uncorr)
***Gard-1 vs.* PBS-1**	mmu00830 Retinol metabolism	2.6486	4.42E-03
	mmu00591 Linoleic acid metabolism	2.4315	8.55E-03
	mmu03010 Ribosome	-8.4850	3.91E-15
	mmu00190 Oxidative phosphorylation	-4.7135	2.03E-06
	mmu04060 Cytokine-cytokine receptor interaction	-3.9074	5.39E-05
	mmu04062 Chemokine signaling pathway	-3.8554	7.19E-05
	mmu04612 Antigen processing and presentation	-3.7393	1.36E-04
	mmu03050 Proteasome	-3.0583	1.60E-03
	mmu04145 Phagosome	-2.8300	2.48E-03
	mmu04672 Intestinal immune network for IgA production	-2.8545	2.82E-03
	mmu04660 T cell receptor signaling pathway	-2.7785	3.10E-03
	mmu04650 Natural killer cell mediated cytotoxicity	-2.6919	3.87E-03
	mmu03040 Spliceosome	-2.5963	5.14E-03
	mmu04514 Cell adhesion molecules (CAMs)	-2.5036	6.43E-03
	mmu00480 Glutathione metabolism	-2.3857	1.00E-02
***Gard-2 vs.* PBS-2**	mmu04510 Focal adhesion	6.2557	6.93E-10
	mmu04144 Endocytosis	6.1367	1.25E-09
	mmu04120 Ubiquitin mediated proteolysis	5.3357	1.68E-07
	mmu04151 PI3K-Akt signaling pathway	5.0988	2.34E-07
	mmu04722 Neurotrophin signaling pathway	4.8336	1.74E-06
	mmu04810 Regulation of actin cytoskeleton	4.6898	1.95E-06
	mmu04520 Adherens junction	4.8834	2.46E-06
	mmu04141 Protein processing in endoplasmic reticulum	4.6692	2.53E-06
	mmu04910 Insulin signaling pathway	4.6103	3.45E-06
	mmu04070 Phosphatidylinositol signaling system	4.4558	1.16E-05
	mmu04360 Axon guidance	4.2598	1.64E-05
	mmu04012 ErbB signaling pathway	4.2117	2.92E-05
	mmu04110 Cell cycle	4.1265	3.06E-05
	mmu04728 Dopaminergic synapse	4.0484	3.72E-05
	mmu04668 TNF signaling pathway	4.0322	4.84E-05

signaling, and antigen processing and presentation were downregulated after the first exposure and upregulated after the second. These results demonstrate that *Gardnerella* elicits a dynamic transcriptional response in the bladder.

Differential Gene Expression Suggests a Role for the Immediate Early Response Pathway

It is not atypical for gene set enrichment analyses to uncover more effects than can be detected when assessing significantly differentially expressed individual genes, especially in biologically complex samples like whole organ homogenates. Considering the complexity of the model, which depends on the outcomes of the initial UPEC reservoir formation phase and on the duration of time *Gardnerella* is maintained in the bladder after each inoculation, it was not entirely surprising that few significant differences were detectable when the data were analyzed at the gene level, comparing PBS-1 *vs. Gard*-1 and PBS-2 *vs. Gard*-2. Five genes were significantly increased (FDR adjusted $P < 0.05$, $\log_2FC > 1.6$) following the first *Gardnerella* exposure (**Figure 3** and **Supplemental Table 8**), and no genes withstood FDR correction after the second *Gardnerella* exposure. Notably, all of the upregulated genes (*Atf3*, *Fosb*, *Nur77, Nurr1* and *Arc*) belong to the class of Immediate Early (IE) response genes that are rapidly co-induced in multiple cell types in response to external stimuli, such as infection and inflammatory signals (Wilson et al., 2010; Bahrami and Drablos, 2016; Crean and Murphy, 2021). This suggests that the presence of these genes in the dataset was not random or artifactual, but reflects a coordinated rapid response to *Gardnerella* exposure. The fact that these IE genes were increased at the first time point, but not at the second, is consistent with previous reports demonstrating rapid induction and then return to baseline of IE genes (Pei et al., 2005; Tan et al., 2020).

Nur77 Is Necessary for Gardnerella-Induced UPEC rUTI

The RNA-seq gene-level data suggested that IE genes could play a role in *Gardnerella*-induced UPEC rUTI. The orphan nuclear receptor *Nur77* (also called *Nr4a1*) is expressed early in the IE pathway, acting as a transcription factor for other IE genes including *Atf3* (Yoon et al., 2011; Gao et al., 2016). We used *Nur77*$^{-/-}$ mice (whole-body) to investigate whether RNA-seq findings could translate to rUTI outcomes. We first established latent UPEC reservoirs in wild type C57BL/6 mice and age-matched *Nur77*$^{-/-}$ mice on the C57BL/6 background (Lee et al., 1995). Since the role of Nur77 during UTI has not previously been studied, we examined whether the absence of Nur77 impacted UPEC titers during initial infection (yellow bar on **Figure 1**). There was no significant difference in the overall level of acute UPEC bacteriuria at 24 hours post infection (hpi) during the initial infection between the mouse strains (**Figure 4A**), although the proportion of mice with low titers ($<10^4$ CFU/mL) was significantly greater in *Nur77*$^{-/-}$ compared to WT mice (0% WT, 14% *Nur77*$^{-/-}$; Fisher's exact $P < 0.05$). In a subset of

TABLE 2 | Temporal Dynamics of Pathway Expression Following *Gardnerella* Exposures.

KEGG Pathway	*Gard*-1 *vs.* PBS-1 logFC	*Gard*-2 *vs.* PBS-2 logFC
mmu00830 Retinol metabolism	2.648601	-2.510346
mmu00140 Steroid hormone biosynthesis	1.996097	-1.931811
mmu04060 Cytokine-cytokine receptor interaction	-3.907424	2.897815
mmu04062 Chemokine signaling pathway	-3.855439	3.037386
mmu04612 Antigen processing and presentation	-3.739260	2.294584
mmu04660 T cell receptor signaling pathway	-2.778472	3.455259
mmu04650 Natural killer cell mediated cytotoxicity	-2.691867	3.428895
mmu03040 Spliceosome	-2.596279	3.904566
mmu04514 Cell adhesion molecules (CAMs)	-2.503606	3.139679
mmu00240 Pyrimidine metabolism	-2.328605	2.401758
mmu04640 Hematopoietic cell lineage	-2.206054	1.939485
mmu04064 NF-kappa B signaling pathway	-2.181243	3.326770
mmu04662 B cell receptor signaling pathway	-2.040245	3.059671
mmu03013 RNA transport	-1.933156	3.408601
mmu04664 Fc epsilon RI signaling pathway	-1.852960	2.579883
mmu04142 Lysosome	-1.832163	3.212280
mmu00230 Purine metabolism	-1.815900	2.192536
mmu03030 DNA replication	-1.795758	1.852620
mmu03420 Nucleotide excision repair	-1.774988	2.183002
mmu04146 Peroxisome	-1.750474	2.769018

animals, we examined UPEC titers during the initial infection phase in bladder tissue following clearance of bacteriuria at 2 wpi (a measure of intracellular reservoirs) and there was no significant difference between WT and *Nur77*$^{-/-}$ mice (**Figure 4B**). The proportion of mice that had chronic bacteriuria that persisted at > 10^4 CFU/mL urine at all time points during the initial infection phase out to 4 wpi was indistinguishable between the two groups (2/39 WT, 2/67 *Nur77*$^{-/-}$) (**Figure 4C**). Together these data demonstrate that Nur77 is not essential for initial UPEC UTI in naïve mice.

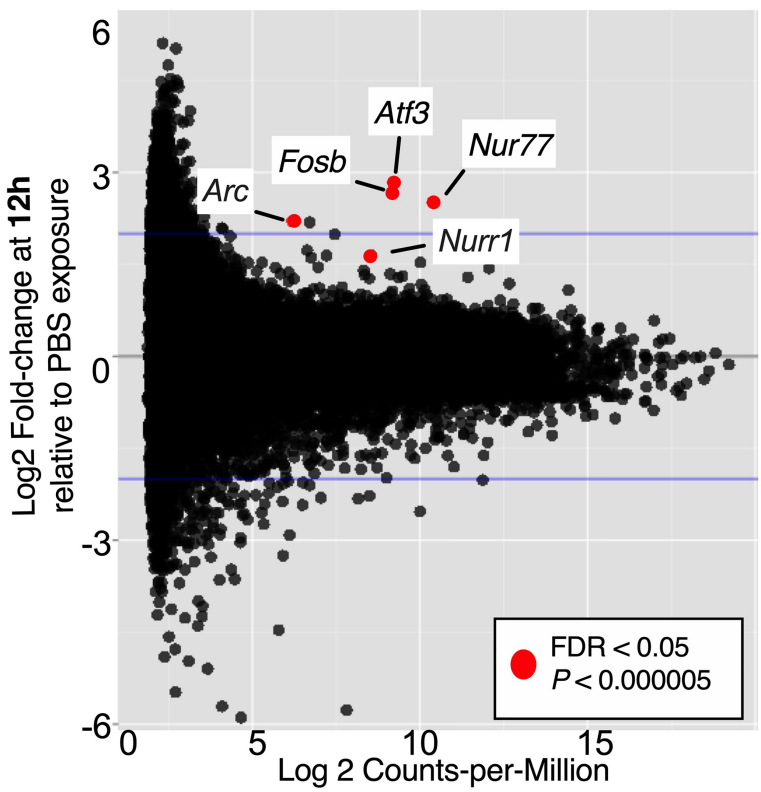

FIGURE 3 | Immediate Early genes upregulated in bladders 12 h after one *Gardnerella* exposure. The volcano plot indicates genes that had significantly higher expression in *Gard*-1 compared to PBS-1 bladders. FDR, false discovery rate correction for multiple comparisons.

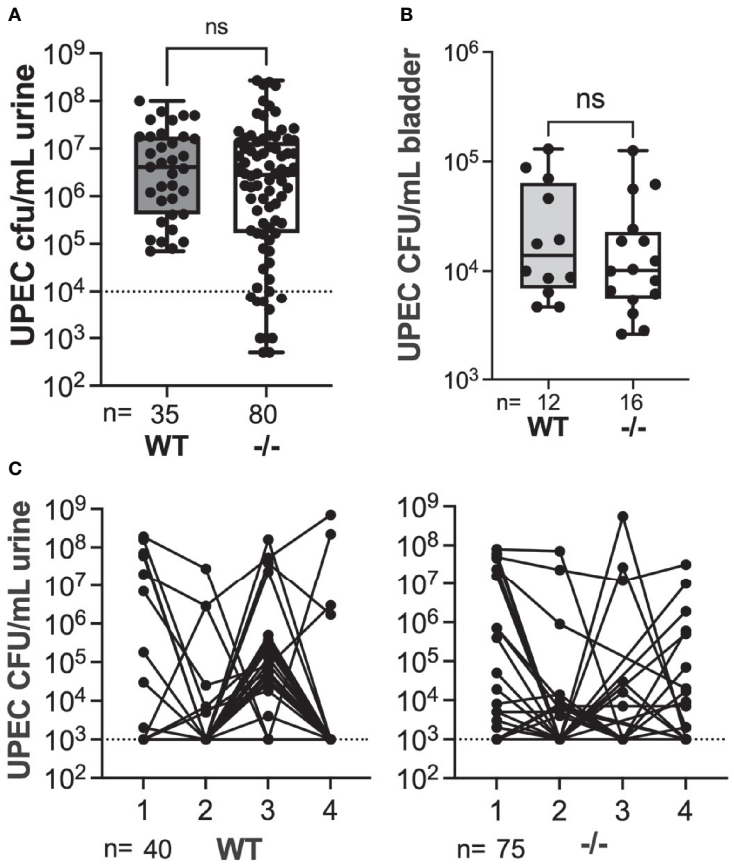

FIGURE 4 | Absence of Nur77 does not affect acute or chronic UPEC bacteriuria or bladder reservoir titers. **(A)** Acute UPEC bacteriuria 24 hpi following initial infection in wild type (WT) mice and mice globally lacking *Nur77* (-/-). **(B)** UPEC titers in bladders collected 2 weeks after initial UPEC infection. All mice had cleared UPEC bacteriuria prior to bladder analysis, thus titers represent intracellular reservoirs. **(C)** UPEC weekly bacteriuria titers following initial UPEC infection. Each dot represents data from an individual mouse. ns, not significant.

Next, we took mice that had cleared initial UPEC bacteriuria by 4 wpi and gave them two secondary exposures, 1 week apart, to *Gardnerella* or PBS. We examined bacteriuria during the subsequent 72 hr after the second exposure as we described previously (Gilbert et al., 2017; O'Brien et al., 2020). We used this exposure model (rather than two exposures 12 h apart, as in the RNA-seq experiment) because it has a higher overall rate of rUTI and because the effects of *Gardnerella* exposure are also evident in the bladder UPEC titers at the experimental endpoint. Similar to our previous studies (Gilbert et al., 2017), the rate of spontaneous emergence in WT PBS control animals was 11% (**Figure 5A**). Although knockout mice appeared to have a somewhat higher baseline of emergence compared to WT, there was no statistically significant difference in rUTI incidence between WT and *Nur77*$^{-/-}$ mice exposed to PBS (11% WT *versus* 26% *Nur77*$^{-/-}$; P = 0.247, Fisher's exact test). Consistent with our previous results, *Gardnerella* exposure increased the incidence of UPEC rUTI in WT mice by approximately 5-fold (56% *Gard vs.* 11% PBS in WT mice; P < 0.01). Conversely, the incidence of rUTI was indistinguishable between the *Gardnerella* and PBS exposure groups in *Nur77*$^{-/-}$ mice 32% *Gard vs.* 29% PBS, **Figure 5A**). In line with our previous findings, *Gardnerella*-exposed WT mice had lower bladder UPEC burdens at the experimental endpoint (**Figure 5B**). This is presumably due to egress of UPEC from the tissue concordant with development of bacteriuria. In contrast, in *Nur77*$^{-/-}$ mice, bladder titers were no different between mice exposed to *Gardnerella* and those exposed to PBS, which is consistent with the urine titer data. Taken together, these results corroborate the RNA-seq data and point to Nur77 as a necessary host factor for *Gardnerella*-induced UPEC rUTI from bladder reservoirs.

DISCUSSION

Millions of women suffer from recurrent UPEC UTI (Foxman et al., 2000; Foxman, 2014), most commonly caused by the same strain that caused the initial infection (Ejrnaes et al., 2006; Czaja et al., 2009; Skjot-Rasmussen et al., 2013; Koljalg et al., 2014). At least some instances of same-strain rUTI are likely due to UPEC emergence from protected bladder intracellular reservoirs

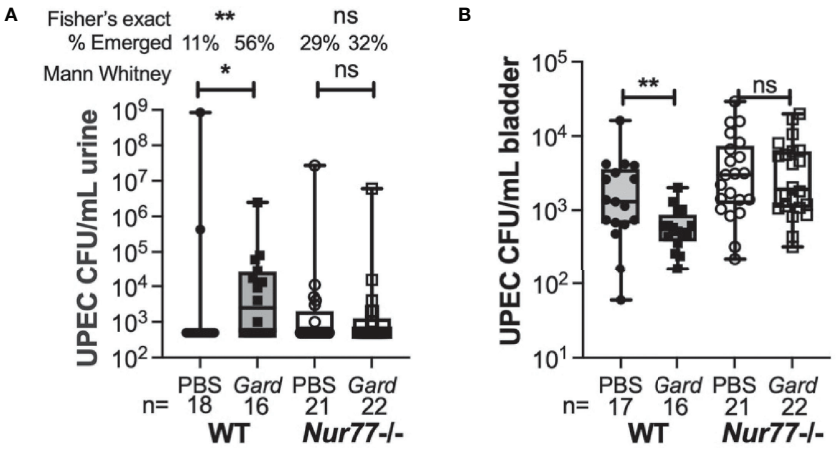

FIGURE 5 | Mice lacking Nur77 are protected from *Gardnerella*-induced UPEC reservoir emergence. Mice harboring bladder UPEC reservoirs were exposed to PBS or *Gardnerella* twice at a one-week interval. Urines were collected daily for three days after the second exposure and mice were euthanized after three days. **(A)** Shown are the highest UPEC urine titers of each individual mouse. **(B)** Bladder UPEC titers are shown. The number of mice in each group is given at the bottom of the graphs. *P < 0.05; **P < 0.01.

(Mulvey et al., 2001; Hickling and Nitti, 2013; Glover et al., 2014). We and others have demonstrated that UPEC can persist intracellularly in the mouse bladder for weeks or months, and can emerge and elicit rUTI after bladder exfoliation is experimentally induced (Mulvey et al., 2001; Schilling et al., 2002; Eto et al., 2006; Mysorekar and Hultgren, 2006). In our model, we use two *Gardnerella* exposures as the trigger for bladder exfoliation and rUTI (Gilbert et al., 2017; O'Brien et al., 2020). In the present study we demonstrate that *Gardnerella* exposures in mice containing UPEC reservoirs affected the expression of host pathways related to urothelial exfoliation and regeneration, mucosal inflammation and immunity, and other processes. Immediate Early (IE) genes were upregulated following one *Gardnerella* exposure. The orphan nuclear receptor Nur77 is a key IE gene (Yoon et al., 2011; Gao et al., 2016), and we found that whole-body *Nur77* knockout mice were protected from recurrent UPEC UTI following *Gardnerella* exposure – that is to say, the rate of rUTI was not increased in *Nur77*$^{-/-}$ mice exposed to *Gardnerella* compared to those mice exposed to PBS. Thus, the IE response may contribute to *Gardnerella*-induced recurrent UPEC UTI.

Gardnerella has frequently been isolated as the most abundant organism in "urinary microbiome" studies, including those using expanded culture techniques to detect live bacteria (Wolfe et al., 2012; Pearce et al., 2014; Hilt et al., 2014; Pearce et al., 2015; Karstens et al., 2016; Wu et al., 2017). Importantly, these studies collected urine by catheterization or suprapubic needle aspiration to limit vaginal contamination of urine specimens, supporting the conclusion that *Gardnerella* can indeed be found within the bladder. In a recent longitudinal culturomics study of healthy women, *Gardnerella* was often present at high relative abundance in urine sample pairs collected from the same woman at different time points (Ksiezarek et al., 2021). Another large study found hospital inpatients with *G. vaginalis* in their urine were more likely to have a history of rUTI than patients in whom *G. vaginalis* was not detected (Josephson et al., 1988). Although these clinical studies make it clear that *Gardnerella* can be found in the bladder and urine, it is not yet known whether *Gardnerella* stably colonizes the urinary tract, *vs.* being repeatedly re-introduced into the bladder by hygienic and/or sexual behaviors. We have shown that *Gardnerella* does not stably colonize the mouse bladder and is cleared within 12 h of exposure, but nonetheless triggers exfoliation and UPEC rUTI (Gilbert et al., 2017). Here we detected significant changes in expression of several pathways even relatively early after a single *Gardnerella* exposure; more substantial changes occurred after two *Gardnerella* exposures as indicated by significant changes in GO terms and KEGG pathways. These findings further support a model in which repeated introduction of *Gardnerella* into the bladder (which may occur in women after sexual activity) can drive host responses and rUTI phenotypes. Thus, our study provides further support for "covert pathogenesis," the notion that a brief exposure to a microbe (here, *Gardnerella*) can affect the host enough to drive disease attributed to another microbe (here, *E. coli*) (Gilbert and Lewis, 2019).

Several pathways identified in this RNA-seq analysis are corroborated by phenotypic observations that we previously reported in this mouse exposure model. As would be expected following bacterial exposure, here we observed changes in many host pathways and processes related to the mucosal immune response. The conclusion that *Gardnerella* exposures trigger an inflammatory response in bladders harboring UPEC reservoirs is further supported by our previous finding of higher levels of IL-12, IFN-g, and RANTES in bladder homogenates (Gilbert et al., 2017). Interestingly, pathways related to T and B cells were upregulated after the second *Gardnerella* exposure. This

observation could implicate adaptive immunity generated by the initial UPEC infection as a contributor to UPEC reservoir emergence. A previous study showed that an adaptive immune response is necessary to clear a challenge UPEC infection that was introduced by a second UPEC inoculation two weeks after a primary UPEC infection in C57BL/6 mice (Mora-Bau et al., 2015). Other studies have demonstrated that bladder inflammation differs between first and second UPEC exposures and that severe bladder inflammation can impact UTI outcomes (O'Brien et al., 2018; Yu et al., 2019). More work is needed to understand bladder immune responses to *Gardnerella* exposure and UPEC reservoir emergence. The RNA-seq pathway analysis also implicated urothelial integrity and turnover, as well as apoptosis. Our previous studies corroborate these findings, as we have shown that our model strain, *Gardnerella* JCP8151B (also used in the present study), elicits both vaginal (Gilbert et al., 2013; Gilbert et al., 2019) and urothelial exfoliation (Gilbert et al., 2017). In the same urinary tract exposure model used here, mice exposed to *Gardnerella* displayed evidence of apoptosis in the urothelium. Compared to mice exposed to PBS, those exposed to *Gardnerella* had increased urothelial TUNEL staining and cleaved Caspase-3 12 h after two *Gardnerella* exposures (Gilbert et al., 2017). Thus, the RNA-seq findings of pathways related to inflammation and urothelial integrity echo the biological phenotypes we previously reported in the mouse model and the findings from our whole organ analysis support the relevance of RNA-seq methods for identifying biologically relevant host responses to *Gardnerella* bladder exposures.

The model and data presented here have some limitations. Although whole bladder RNA-seq is an established method for investigating host response during UTI (O'Brien et al., 2016; Yu et al., 2019), transcriptional changes cannot be attributed to specific cell types, but rather reflect changes that occurred at the organ level. As well, using the whole organ for bulk RNA-seq may mask gene expression changes that only occur in a small subset of cells, which could be one explanation for the relatively modest number of genes for which expression changes after *Gardnerella* exposure were statistically significant following FDR correction. Nonetheless, the RNA-seq data corroborated previously reported phenotypic findings in this model (apoptosis, inflammatory cytokines) and identified a host gene (*Nur77*) that we subsequently found to be necessary for *Gardnerella*-induced rUTI. These findings support the utility of RNA-seq methods for assessing host responses to even transient bladder microbial exposures and warrant future single-cell transcriptomic studies to specifically examine urothelial, immune, and other bladder cell responses.

The model presented here involves multiple exposures to two different microbes that could independently or synergistically stimulate host responses over time. While this likely reflects the situation occurring in women, it does present some challenges for interpretation. A caveat of the current dataset is that it cannot distinguish changes that required a second *Gardnerella* exposure from changes that resulted from the first exposure, but required additional time to become apparent. However, since we previously reported that a single *Gardnerella* exposure was rapidly cleared and did not cause urothelial exfoliation or UPEC rUTI, it is very likely that most of the pathway upregulation observed here was due to the second exposure, but this should be determined in future studies. Our finding that host gene expression pathways were primarily downregulated after one *Gardnerella* exposure and upregulated after two exposures could suggest that the second time point reflects a host genetic signature of UPEC reservoir reactivation. Additionally, since all of the mice used in the present study harbored UPEC reservoirs (because of our interest in rUTI), we cannot distinguish changes that were due specifically and solely to *Gardnerella* from changes that resulted from "re-awakening" of UPEC reservoirs. Relatively little is understood with respect to how UPEC emerge from quiescent intracellular reservoirs and what host processes are involved. Furthermore, UPEC infection has a lasting effect on the bladder mucosa that is likely to also impact how the bladder responds to subsequent exposures. Consistent with this idea, we previously reported that the bladder cytokine changes caused by *Gardnerella* were distinct between naive mice and those with UPEC reservoirs (Gilbert et al., 2017). Future studies aimed at distinguishing between these various possibilities could examine the bladder transcriptome following *Gardnerella* exposure in naive mice and following induction of UPEC rUTI from reservoirs by other means (e.g. protamine sulfate (Mysorekar and Hultgren, 2006) or chitosan (Blango et al., 2014)).

Our RNA-seq data implicated the nuclear receptor Nur77 (aka Nr4a1) as an early responder to *Gardnerella* exposure. Nur77 regulates myriad cellular processes, that intersect with the UPEC UTI pathogenic cascade and could influence rUTI outcomes. For example, Nur77 regulates apoptosis in multiple tissue types (Rajpal et al., 2003; Herring et al., 2019), and we previously found that *Gardnerella* induced bladder exfoliation *via* apoptosis (Gilbert et al., 2017). Whether or not Nur77 drives exfoliation in the *Gardnerella*-exposed urothelium remains to be determined. Nur77 also modulates inflammation (Rodriguez-Calvo et al., 2017) and has been specifically implicated in T cell responses (Liebmann et al., 2018) and Ly6C- monocytes (Hanna et al., 2011). Nur77 modulated inflammatory responses to *E. coli* in the lung during pneumonia in mice (Cui et al., 2019), but the role of Nur77 in mediating bladder responses to UPEC has not been examined. In the present study we did not conduct an in-depth characterization of acute UPEC UTI in *Nur77*$^{-/-}$ mice. However, the first phase of our rUTI model entails UPEC infection and monitoring of urine bacterial titers over time during the initial infection phase, prior to secondary exposures. We observed no differences between wild type and *Nur77*$^{-/-}$ mice in the initial UPEC infection phase of the model. As well, we found that *Nur77*$^{-/-}$ mice did harbor stable bladder UPEC reservoirs. Thus, *Nur77* may be dispensable for initial UPEC bladder infection in C57BL/6 mice, though we did not assess chronic bladder or kidney infection or other mouse UTI phenotypes. Strikingly, we found that *Nur77*$^{-/-}$ mice were protected from *Gardnerella*-induced recurrent UPEC UTI, as evidenced by no difference in the incidence of post-exposure bacteriuria or remaining bladder reservoir titers between

Gardnerella-exposed *vs.* PBS-exposed *Nur77*$^{-/-}$ mice. In this pilot study, the wild type and *Nur77*$^{-/-}$ mice were not littermates. Future studies will directly compare *Nur77*$^{-/-}$ mice to *Nur77*$^{+/-}$ and wild type littermate controls to validate our rUTI findings. Nur77 is a druggable target, with several ligands being explored for treatment of diseases such as cancer, metabolic disorders, hyperinflammatory responses and endometriosis (Wu and Chen, 2018; Mohankumar et al., 2020). If our findings translate to rUTI in humans, this could open up a therapeutic avenue that is much needed in the current climate of increasing antibiotic resistance (Tamadonfar et al., 2019). Future studies will more closely examine the mechanism(s) for Nur77-mediated response to *Gardnerella* in the bladder, will investigate whether IE responses impact UTI phenotypes in other mouse models, and will test whether Nur77 could also play a role in host response to *Gardnerella* in the vagina.

MATERIALS AND METHODS

Ethics Statement

Mouse experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee (IACUC) of Washington University School of Medicine approved the protocol (Protocol Number: 20170081).

Bacterial Strains and Growth Conditions

Uropathogenic *E. coli* strain UTI89, harboring a kanamycin resistance cassette (Wright et al., 2005), was grown aerobically at 37°C in static liquid culture in Lysogeny Broth (LB) medium, or on LB agar plates with 25 μg/ml kanamycin. *Gardnerella* strain JCP8151B (Lewis et al., 2013), historically regarded as *G. vaginalis* and recently referred to as *G. piotii* (Hill et al., 2019), was grown anaerobically at 37°C in shaking liquid culture in NYCIII medium, or on NYCIII agar plates with 1 mg/ml streptomycin. Mouse inocula were prepared as previously described (O'Brien et al., 2020).

Mice

Six- to seven-week-old female C57BL/6 mice ("wild type") were obtained from Charles River (Fredericks facility). Mice globally deficient in *Nur77* (a.k.a. Nr4a1) were obtained from Jackson Laboratories (B6;129S2-Nr4a1tm1Jmi/J, catalog #006187). Mice were given a regular chow diet in a specific pathogen-free facility with a 12 h light/12 h dark cycle at Washington University School of Medicine. Mice were allowed to acclimate to the facility after transport for 1 week prior to experiments.

Mouse Urinary Tract Inoculation Experiments for RNA-Seq

Experiments were performed essentially as described previously (Gilbert et al., 2017; O'Brien et al., 2020). Briefly, mice were anesthetized with isoflurane and then inoculated transurethrally with 50 μL prepared 1 x 10^7 CFU UPEC inoculum. Urine was collected at 24 hpi, and weekly thereafter for 4 weeks. Mice that no longer had detectable UTI89 in urine at 4 weeks, reflecting resolution of the initial bladder lumen infection, were then inoculated transurethrally with 50 uL prepared inoculum of 1 x 10^8 CFU *Gardnerella* strain JCP8151B (10 mice) or PBS (10 mice). Twelve hours later, five mice from each exposure group (*Gard*-1 and PBS-1) were sacrificed and their bladders were collected aseptically and flash frozen in liquid nitrogen for future RNA isolation. The remaining five mice per group received a second transurethral inoculation with JCP8151B or PBS and were sacrificed another 12 h later to collect bladders for RNA isolation (*Gard*-2 and PBS-2).

Library Preparation and Sequencing

Bladders were homogenized and RNA was extracted using the RNeasy Plus Mini kit (Qiagen). Libraries were prepared from each bladder individually with 10 ng of total RNA and RNA integrity was determined using an Agilent Bioanalyzer, with a Bioanalyzer RIN score greater than 8.0 obtained for all samples. ds-cDNA was prepared using the SMARTer Ultra Low RNA kit for Illumina Sequencing (Takara-Clontech) per the manufacturer's protocol. cDNA was fragmented using a Covaris E220 sonicator using peak incident power 18, duty factor 20%, cycles/burst 50, time 120 seconds to yield an average size of 200 base pairs (bp). cDNA was then blunt ended, had an A base added to the 3' ends, and then had Illumina sequencing adapters ligated to the ends. Ligated fragments were then amplified for 12 cycles using primers incorporating unique index tags. Fragments were multiplexed with 5-6 samples per lane and were sequenced on an Illumina HiSeq 2500 using single end 50 bp reads to target 30M per sample.

RNA-Seq Data Acquisition, Quality Control, and Processing

RNA-seq reads from the twenty individual libraries (5 mice per exposure group) were demultiplexed using a custom demultiplexing script written in Python and then aligned to the Ensembl GRCm38.76 (*Mus musculus*) assembly with STAR version 2.0.4b. Gene counts were derived from the number of uniquely aligned unambiguous reads by Subread:featureCount version 1.4.5. Transcript counts were produced by Sailfish version 0.6.3. Sequencing performance was assessed for total number of aligned reads, total number of uniquely aligned reads, genes and transcripts detected, ribosomal fraction, known junction saturation and read distribution over known gene models with RSeQC version 2.3. All gene-level and transcript counts were then imported into the R/Bioconductor package EdgeR and TMM-normalized to adjust for differences in library size. Genes or transcripts not expressed in any sample were excluded from further analysis. Performance of the samples was assessed with a Spearman correlation matrix and multi-dimensional scaling plots. Generalized linear models with robust dispersion estimates were created to test for gene/transcript level differential expression. The fits of the trended and tagwise dispersion estimates were then plotted to confirm proper fit of the observed mean to variance relationship where

the tagwise dispersions are equivalent to the biological coefficients of variation of each gene. Differentially expressed genes and transcripts (comparing PBS-1 *vs. Gard*-1 and PBS-2 *vs. Gard*-2) were then filtered for FDR adjusted P values less than or equal to 0.05. For each EdgeR contrast, global perturbations in known Gene Ontology (GO) terms and KEGG pathways were detected using the R/Bioconductor package GAGE to test for changes in expression of the reported $\log_2$ fold-changes reported by edgeR in each term *versus* the background $\log_2$ fold-changes of all genes found outside the respective term. The R/Bioconductor package heatmap3 was used to display heatmaps across groups of samples for each GO term with a Benjamini-Hochberg false-discovery rate adjusted P value less than or equal to 0.05.

Recurrent UTI Experiments in Wild Type and *Nur77* -/- Mice

Mice were anesthetized with isoflurane and then inoculated transurethrally with 50 µL prepared UPEC inoculum. Urine was collected at 24 hpi, and weekly thereafter for 4 weeks. A subset of mice were sacrificed at 2 weeks to compare bladder reservoir titers. Mice that no longer had detectable UTI89 in urine at 4 weeks, reflecting resolution of the initial bladder lumen infection, were used for recurrent UTI experiments. The groups were frequency matched based upon the time course of clearance of UPEC urine titers during the initial infection (O'Brien et al., 2020). Mice were given two bladder exposures of PBS or *Gardnerella*, 1 week apart (transurethral inoculations prepared as in experiments described above). Urine was collected at 24, 48 and 72 h after the second exposure and titers were enumerated by serial dilution and plating on selective media (LB+kanamycin to detect UPEC; NYCIII+streptomycin to detect *Gardnerella*). At 72 h, mice were humanely sacrificed *via* cervical dislocation under isofluorane anaesthesia and bladders and kidneys were aseptically harvested. Homogenates were prepared in 1 mL sterile PBS and plated on appropriate selective media. Bacterial burden in each sample was calculated as CFU/bladder. Samples with no colonies were plotted at one-half of the limit of detection.

DATA AVAILABILITY STATEMENT

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE186800 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE186800).

ETHICS STATEMENT

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee (IACUC) of Washington University School of Medicine

AUTHOR CONTRIBUTIONS

NG and VO'B performed experiments. NG, VO'B, and AL analyzed the data. NG and VO'B drafted the manuscript. All authors provided funding to support the research. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.788229/full#supplementary-material

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METHODS
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A Semi-Quantitative Assay to Measure Glycosaminoglycan Degradation by the Urinary Microbiota

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Glycosaminoglycans (GAGs) are linear polysaccharides and are among the primary components of mucosal surfaces in mammalian systems. The GAG layer lining the mucosal surface of the urinary tract is thought to play a critical role in urinary tract homeostasis and provide a barrier against urinary tract infection (UTI). This key component of the host-microbe interface may serve as a scaffolding site or a nutrient source for the urinary microbiota or invading pathogens, but its exact role in UTI pathogenesis is unclear. Although members of the gut microbiota have been shown to degrade GAGs, the utilization and degradation of GAGs by the urinary microbiota or uropathogens had not been investigated. In this study, we developed an *in vitro* plate-based assay to measure GAG degradation and utilization and used this assay to screen a library of 37 urinary bacterial isolates representing both urinary microbiota and uropathogenic species. This novel assay is more rapid, inexpensive, and quantitative compared to previously developed assays, and can measure three of the major classes of human GAGs. Our findings demonstrate that this assay captures the well-characterized ability of *Streptococcus agalactiae* to degrade hyaluronic acid and partially degrade chondroitin sulfate. Additionally, we present the first known report of chondroitin sulfate degradation by *Proteus mirabilis*, an important uropathogen and a causative agent of acute, recurrent, and catheter-associated urinary tract infections (CAUTI). In contrast, we observed that uropathogenic *Escherichia coli* (UPEC) and members of the urinary microbiota, including lactobacilli, were unable to degrade GAGs.

Keywords: urinary tract infection, glycosaminoglycans, urinary microbiota, uropathogenic bacteria, *in vitro* assay, *Proteus mirabilis*, chondroitin sulfate

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INTRODUCTION

Urinary tract infection (UTI) is among the most common adult bacterial infections encountered in community and clinical settings. When a patient experiences ≥2 symptomatic infections in six months or ≥3 infections in one year, it is defined as recurrent urinary tract infection (rUTI) (Malik et al., 2018b). UTIs can emerge from a diverse set of bacterial and fungal pathogens but are

predominantly caused by uropathogenic *Escherichia coli* (UPEC) (Klein and Hultgren, 2020). Present rUTI therapies heavily rely on antimicrobials to achieve sterility in the urinary tract, but are undermined by increasing rates of antimicrobial resistance and allergy (Malik et al., 2018a). In order to develop new therapies for rUTI, more research is needed to understand how the host environment contributes to UTI pathogenesis and recurrence.

Contrary to public perception, the urinary tract is not sterile in healthy individuals (Hilt et al., 2014; Neugent et al., 2020). In fact, it is thought that *Lactobacillus* spp. play a protective role in the urogenital tract as their absence has been associated with various disease states (Amabebe and Anumba, 2018; Price et al., 2020). While a constant flux of urine containing electrolytes, osmolytes, amino acids, and carbohydrates may support the urinary microbiota, an understudied carbon source in the urinary tract is the glycosaminoglycan (GAG) layer lining the luminal surface of the bladder epithelium (**Figure 1A**). GAGs are present in every mammalian tissue and are composed of negatively-charged linear heteropolysaccharides containing repeating disaccharides units composed of uronic acid (or galactose) and amino sugars (Gandhi and Mancera, 2008). Clinically relevant GAGs include heparin/heparan sulfate (HP), chondroitin sulfate (CS), hyaluronic acid (HA), and keratan sulfate (KS) (Casale and Crane, 2021) (**Figure 1B**). All of these GAGs except for HA are covalently bound to core proteins *in vivo* as proteoglycans (Raman et al., 2005). CS, HP, and HA have been detected in human urine and are predicted to comprise the GAG layer of the urinary tract (Sun et al., 2015; Han et al., 2020). Within vertebrate mucosal environments, GAGs provide cell hydration and structural scaffolding but also mediate many crucial biochemical processes, including regulation of cell growth and proliferation, anticoagulation, and wound repair. Also, some pathogenic bacteria produce extracellular capsules comprised of glycosaminoglycans (e.g. the HA capsule of *Streptococcus pyogenes*) that aid in immune evasion and improve host colonization (DeAngelis, 2002).

Species belonging to the genera *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, and *Streptococcus* derived from the human gut have been found to express enzymes (e.g. heparin lyase) that degrade GAGs into smaller, metabolizable disaccharides (Hynes and Walton, 2000; Kawai et al., 2018; Zúñiga et al., 2018). However, the ability of the urinary microbiota and invading uropathogens to metabolize GAGs has not been assessed. One obstacle to screening of GAG degradation and utilization phenotypes in diverse microbial species has been the lack of a fast quantitative or semi-quantitative method to measure their abundance in liquid

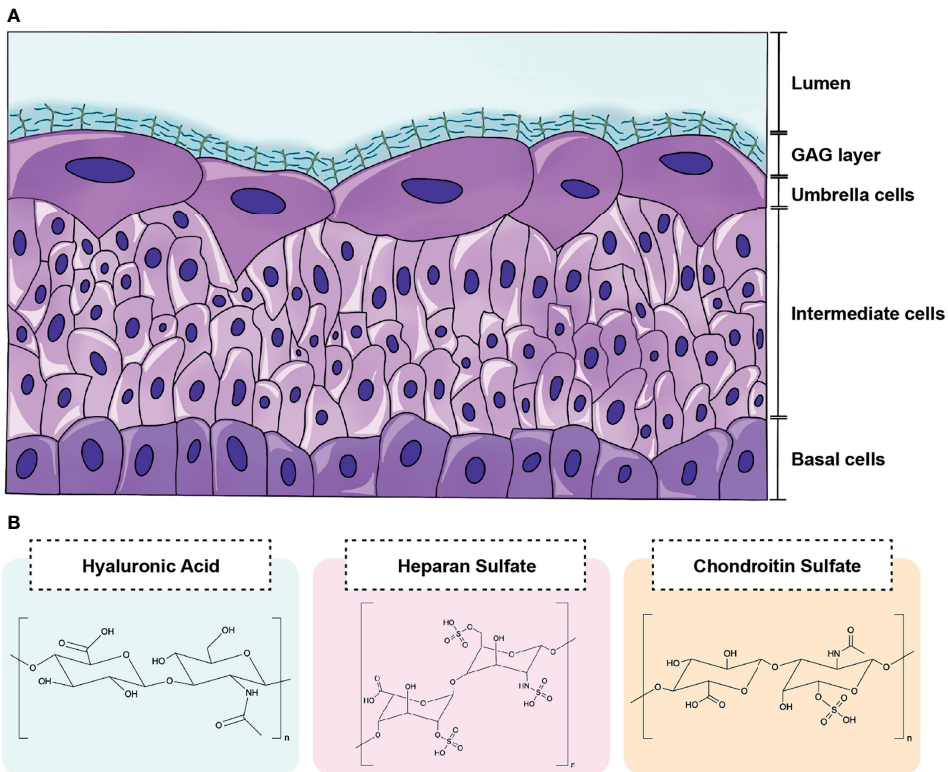

FIGURE 1 | Schematic of GAG layer and major urinary GAG structures. **(A)** The luminal surface the bladder epithelium is coated by a GAG layer that lines terminally differentiated superficial cells termed umbrella cells. **(B)** The GAGs most relevant to the urinary tract include hyaluronic acid (HA), heparin/heparan sulfate (HP), and chondroitin sulfate (CS). HA contains repeating disaccharide units of d-glucuronic acid and N-acetylglucosamine bonded by alternating β-1,4 and β-1,3 linkages. HP is comprised of repeating disaccharide units d-glucuronic acid and d-N-acetylglucosamine bonded by β-1,4 linkages. CS consists of N-acetyl-d-galactosamine and β-glucuronic acid connected by alternating β-1,3 and β-1,4 linkages.

media. The commonly used 1,9-dimethylmethylene blue (DMMB) assay, for example, can only measure sulfated GAGs and therefore cannot measure HA (Barbosa et al., 2003; Zheng and Levenston, 2015). To enable rapid measurement of both sulfated and non-sulfated GAGs in solution, we developed a novel *in vitro* 96-well plate-based, semi-quantitative GAG degradation and growth assay that has increased sensitivity and cost-effectiveness compared to previously reported agar-based screening methods (Kawai et al., 2018). Following assay validation in multiple media types, we then used this assay to screen 37 bacterial strains isolated from the urine of women with different UTI histories for the ability to degrade and utilize CS, HP, and HA in minimal or artificial urine media. We found that while most screened urinary microbiota strains, including *L. crispatus, L. gasseri, L. jensenii, L. johnsonii, L. rhamnosus*, were unable to degrade GAGs and utilize them for growth, the invasive uropathogenic bacterium, *Proteus mirabilis*, efficiently degraded CS in both minimal and artificial urine medium.

METHODS

Bacterial Strains

The bacterial strains used in this study were originally isolated from clean-catch midstream urine collected from consenting postmenopausal women (age 55-85) who were either healthy (no current UTI), or had active, symptomatic rUTI as part of institutional review board-approved studies STU 032016-006 and MR 17-120. Species identification of the 37 urinary bacterial strains used in this study was performed by 16S rRNA PCR Sanger Sequencing as previously described (Sharon et al., 2021). The species used in this study are listed in **Table 1** followed by the number of strains assayed per species. Initial cultivation was performed on anaerobic blood agar plates (BD BBL) in anaerobic atmosphere at 35°C, and liquid cultivation was performed in the media and conditions described in **Table 1**. Most experiments were performed under microaerophilic conditions to mimic atmospheric conditions in the bladder. *Bifidobacterium* spp. are obligate anaerobes and GAG assays were performed under anaerobic conditions. *Pedobacter heparinus* is an obligate aerobe and assays were performed in aerobic atmosphere. All strains were incubated at 35°C except for *P. heparinus*, which grows only at 30°C after 72 hours. Additionally, incubation time was 48 hours for most urinary isolates due to slower doubling times and 24 hours for *Escherichia coli* and *Klebsiella pneumoniae* to prevent overgrowth. *P. heparinus* HIM 762-3 (ATCC), which is an environmental bacterium known to degrade CS, HP, and HA, was used as a positive control while *Escherichia coli* K12 MG1655 was used as a negative control (Kawai et al., 2018). *P. heparinus* was initially cultivated on brain heart infusion (BHI) agar in aerobic atmosphere at 30°C.

Overview of GAG Utilization and Degradation Assay

In this study, bacterial GAG utilization and degradation were assessed using a single, microtiter plate-based assay (**Figure 2**). To assay the ability of urinary bacteria to utilize GAGs for growth, we supplemented 2.5 mg/mL GAGs to a minimal basal media or artificial urine media (AUM) and examined the resulting optical density measurements compared to glucose controls (**Tables 1** and **S1**). To investigate if urinary bacteria degraded GAGs, we subsequently developed and performed a novel semi-quantitative microtiter assay that leverages the precipitation of bovine serum albumin (BSA)-complexed GAGs by acetic acid (**Figure 2A**) to allow determination of the percentage of GAG remaining by optical density (OD) measurement. Furthermore, we used known concentrations of GAGs to establish standard curves relating OD_{600} to GAG

TABLE 1 | Strain list and experimental conditions.

Bacteria	No. of Strains	Pre-culture Media	Basal Media	AUM Media	Atmospheric Condition	Incubation Time (hrs)
Bifidobacterium breve (BB)	1	MRS^{L}	$mMRS^{L}$	AUM^{YL}	Anaerobic	48
Bifidobacterium longum (BL)	1	MRS^{L}	$mMRS^{L}$	AUM^{YL}	Anaerobic	48
Enterococcus faecalis (EF)	3	BHI	$M9^{YC}$	AUM^{Y}	Microaerophilic	24
Escherichia coli (EC)	4	BHI	M9	AUM	Microaerophilic	24
Klebsiella pneumoniae (KP)	3	BHI	M9	AUM	Microaerophilic	24
Lactobacillus crispatus (LC)	3	MRS	mMRS	AUM^{YT}	Microaerophilic	48
Lactobacillus gasseri (LG)	3	MRS	mMRS	AUM^{YT}	Microaerophilic	48
Lactobacillus jensenii (LJe)	2	MRS	mMRS	AUM^{YT}	Microaerophilic	48
Lactobacillus johnsonii (LJo)	1	MRS	mMRS	AUM^{YT}	Microaerophilic	48
Lactobacillus rhamnosus (LR)	2	MRS	mMRS	AUM^{YT}	Microaerophilic	48
Proteus mirabilis (PM)	4	BHI	$M9^{Y}$	AUM	Microaerophilic	48
Staphylococcus epidermidis (SE)	2	BHI	mMRS	AUM^{Y}	Microaerophilic	48
Streptococcus agalactiae (SA)	3	BHI	$M9^{Y}$	AUM^{Y}	Microaerophilic	48
Streptococcus anginosus (SAn)	2	BHI	mMRS	AUM^{Y}	Microaerophilic	48
Streptococcus oralis (SO)	1	BHI	mMRS	AUM^{Y}	Microaerophilic	48
Streptococcus parasanguinis (SPa)	1	BHI	mMRS	AUM^{Y}	Microaerophilic	48
Streptococcus pneumoniae (SPn)	2	BHI	mMRS	AUM^{Y}	Microaerophilic	48
Pedobacter heparinus (PH)	1	BHI	YE0.1	–	Aerobic	72

[C]Casamino Acids; [L]L-cysteine hydrochloride; [Y]Yeast Extract; [T]Tween 80.

A total of 37 urinary strains representing 17 species were screened. Pre-culture media was used for overnight growth in preparation for the GAG growth and degradation assays. Experimental conditions for GAG growth and degradation assays in basal and artificial urine media. Optimized basal and AUM media for each species are listed and exact formulations are listed in ***Table S1****.*

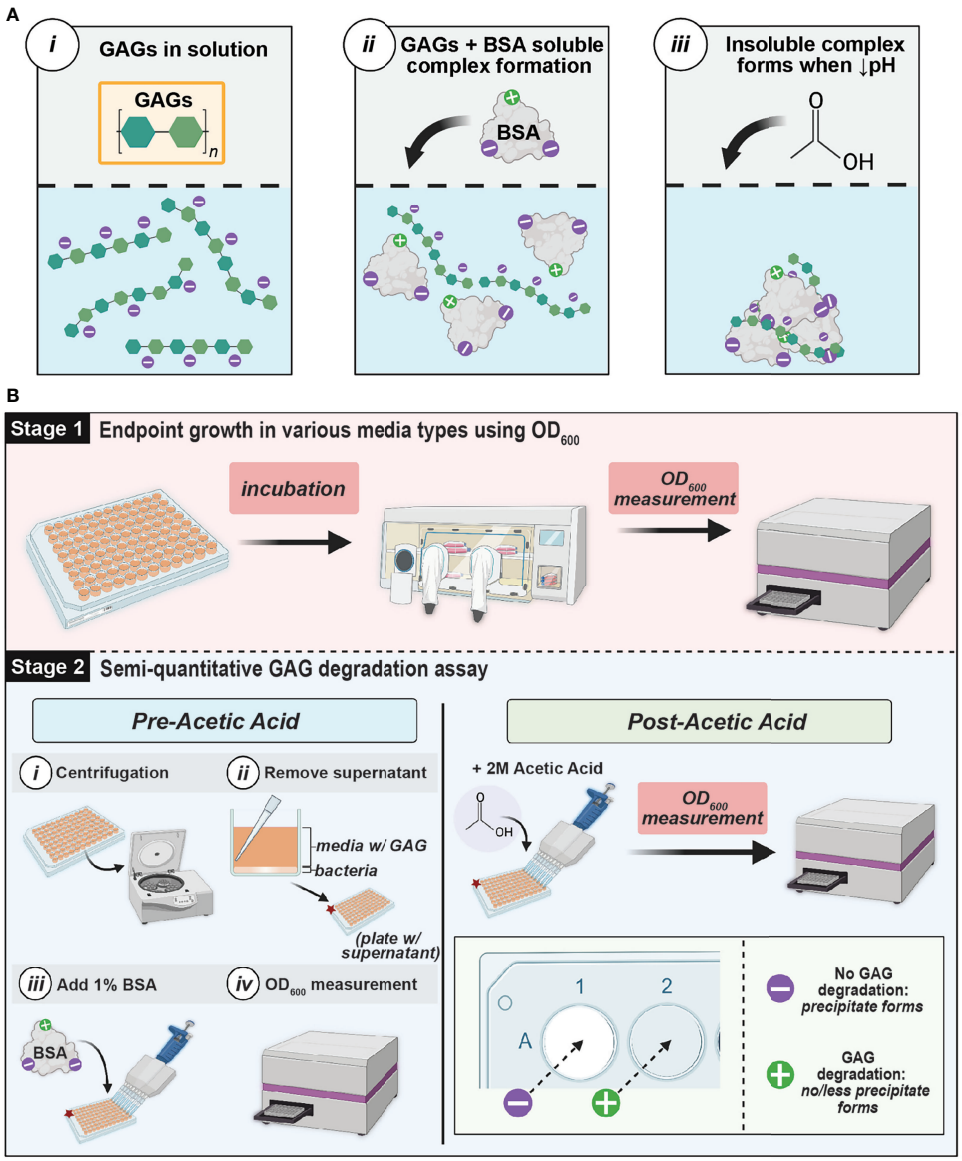

FIGURE 2 | GAG assay theory and workflow **(A)** Schematic of interaction between GAGs and BSA. GAGs and BSA form a soluble complex in solution (ii), but a decreased pH causes an insoluble complex to form and precipitate (iii) **(B)** GAG Growth and Semiquantitative Assay Method Workflow. Stage 1 involves measuring the endpoint growth of bacteria in media in the presence of GAGs versus glucose. Stage 2 evaluates GAG degradation by leveraging the precipitation of BSA complexed with remaining GAGs in the media supernatant at low pH. If there is GAG remaining after incubation, a white precipitate will form. However, if complete GAG degradation occurred, there will be no precipitate formation. The extent of GAG-BSA precipitation can be determined by measuring the optical density at 600 nm (OD_{600}). This figure was created using BioRender.com.

concentration in 10 different media to allow estimation of the concentration of GAGs remaining in solution within an established linear range.

GAGs and Glucose Stock Solution Preparation

All GAG (HA, HP, and CS) and glucose stock solutions were prepared in Milli-Q water and sterilized by passing through a 0.2µm cellulose acetate membrane filter (VWR). Hyaluronic acid (Sigma-Aldrich; Cat. No. 53747) was prepared at a concentration of 5g/L and sonicated (Branson Ultra Sonic Bath Model 3800) at 37°C for 1 hour to aid in solubilization. Heparin sodium (Fisher BioReagents; Cat. No. BP2425) and chondroitin sulfate A sodium salt (Sigma-Aldrich; Cat. No. C9819) solutions were prepared at a concentration of 10g/L. Glucose (Fisher Chemical; Cat. No. D16) was dissolved and stored at a 200g/L stock concentration. For plate-based assays, GAGs and glucose were supplemented to all media at a final concentration of 2.5mg/mL.

Influence of GAGs on Microbial Growth Using Endpoint Optical Density Measurements

All bacterial strains were precultured using either De Man, Rogosa, and Sharpe Media (MRS) (BD Difco™; Cat. No. DF0881-17-5), MRS^{L}, or Brain Heart Infusion (BD Difco™, BHI; Cat. No. DF0418-17-7) under various atmospheric conditions and incubation times listed in **Table 1** at 35°C with the exception of *P. heparinus* which was cultivated at 30°C. BD GasPak EZ anaerobe pouch systems (Cat. No. 260683) were used for anaerobic culture and BD GasPak EZ CampyPouch systems (Cat. No. 260685) were used for microaerophilic incubation. After pre-culturing, optical density measurements at 600nm (OD_{600}) were taken using a BioTek™ Synergy™ H1 plate reader. Cultures were then normalized to an OD_{600} = 0.05 using sterile 1X phosphate-buffered saline (PBS) and centrifuged at 3381 x g for 10 minutes. Supernatant was removed and pellets were washed using sterile 1X PBS and resuspended in the appropriate basal or AUM media with 2.5 mg/mL HA, HP, CS, glucose, or unsupplemented. After resuspension, technical replicates were placed in 96-well microtiter plates and incubated for the time periods and in the atmospheric conditions specified in **Table 1**. Post-incubation OD_{600} measurements were taken and normalized to bacteria-free controls to determine if growth occurred in each condition (**Figure 2B**).

Semi-Quantitative GAG Degradation Measurement

A 96-well plate-based semi-quantitative assay was performed to examine GAG degradation and is illustrated in **Figure 2**. After OD_{600} growth measurements were taken, the 96-well microtiter plates were centrifuged at 3214 x g for 10 minutes to pellet bacteria. The supernatant was then transferred to a microtiter plate and two-fold serial dilutions were performed in 1X PBS to a final volume of 90µL in each well. Molecular-biology grade bovine serum albumin (BSA) (Fisher Scientific; Cat. No. 50-550-390) was then added to a final concentration of 1% to the dilutions and OD_{600} measurements were recorded (pre-acetic acid values). BSA and GAGs form a soluble complex in solution (Hattori et al., 2001) (**Figure 2A**). 40µL of 2M acetic acid (Sigma-Aldrich; Cat. No. 1000631011) was then added and stirred using a pipette tip. OD_{600} measurements were immediately taken (post-acetic acid values). In the presence of acetic acid, the GAG and BSA complex will form a white, insoluble precipitate. If the solution is completely white, no GAG degradation has occurred. However, if less precipitate forms or the solution is less white or completely clear, then GAG degradation has occurred (**Figure 2B**). A semi-quantitative calculation can be performed to estimate the percentage of GAG remaining:

$$\frac{(Bacteria)\ Post\ Acetic\ Acid\ OD_{600} - Average\ of\ Pre\ Acetic\ OD_{600}}{(Control)\ Post\ Acetic\ Acid\ OD_{600} - Average\ of\ Pre\ Acetic\ OD_{600}} \times 100$$

To select the appropriate dilutions of OD_{600} inputs for the semi-quantitative calculation, we generated standard curves of bacteria-free controls containing known concentrations of each GAG. Dilutions within the 95% confidence interval (CI) of the standard curve were selected for quantitation. Further, we used these experimentally generated standard curves to quantitate the amount of GAG remaining. GAG concentrations were interpolated by simple linear regression of the standard curves. All dilutions used for both semi-quantitative and quantitative GAG measurements were within the linear range and 95% CI of the respective standard curve.

Statistical Analyses and Chemical Structures

All statistical analyses were performed with GraphPad Prism Version 9.2.0. One-way ANOVA with Dunnett's multiple comparisons *post-hoc* was used for hypothesis testing. An α of 0.05 was considered significant to control for type I error. Chemical structures were generated using ChemDraw Version 19.0.0.26.

RESULTS

Standard Curve and Linear Dynamic Range

Standard curves were generated using serial dilutions of known concentrations of HA, HP, and CS in 10 media types (**Figure 3** and **Figures S1, S2**) to determine limit of detection and define a linear range for semi-quantitative and quantitative GAG measurements. Concentrations of each GAG were plotted against the corresponding OD_{600} readout values and linear regression analysis was performed. 95% confidence intervals were determined and used to define the linear dynamic range of the assay for each GAG in each media type. The standard curves for HA, HP, and CS in basal medium M9 and AUM are depicted in **Figures 3A, B**, respectively. For M9 and AUM, the linear dynamic range was 0.0391mg/ml-0.625mg/mL for HA, 0.313mg/mL-1.25mg/mL for HP, and 0.0781mg/mL-0.625mg/mL CS (**Figures 3A, B**). In the case of M9^{YC}, mMRS, mMRS^{L}, YE0.1, AUM^{YL}, AUM^{YT}, the linear dynamic range was 0.625mg/mL-2.50mg/mL for HP (**Figures S1, S2**). Values within the linear dynamic range were used to calculate semi-quantitative GAG concentrations and interpolate GAG concentration quantitative measurements.

Assay Validation

GAG degradation assay specificity and sensitivity was evaluated using bacterial species with well-defined GAG degradation activity. *Pedobacter heparinus* has been shown to degrade HA, HP, and CS, while *Escherichia coli* K12 demonstrates no GAG degradation activity (Hashimoto et al., 2014; Kawai et al., 2018). *Streptococcus agalactiae* is known to both degrade and metabolize HA, but can also degrade CS due to the similar substrate specificities of hyaluronate lyase (Stern and Jedrzejas, 2006). However, the catabolism of CS by hyaluronate lyase is slower due to sulfation patterns absent in HA (Li and Jedrzejas, 2001; Stern and Jedrzejas, 2006). Semi-quantitative GAG degradation assays were performed using *P. heparinus* (PH),

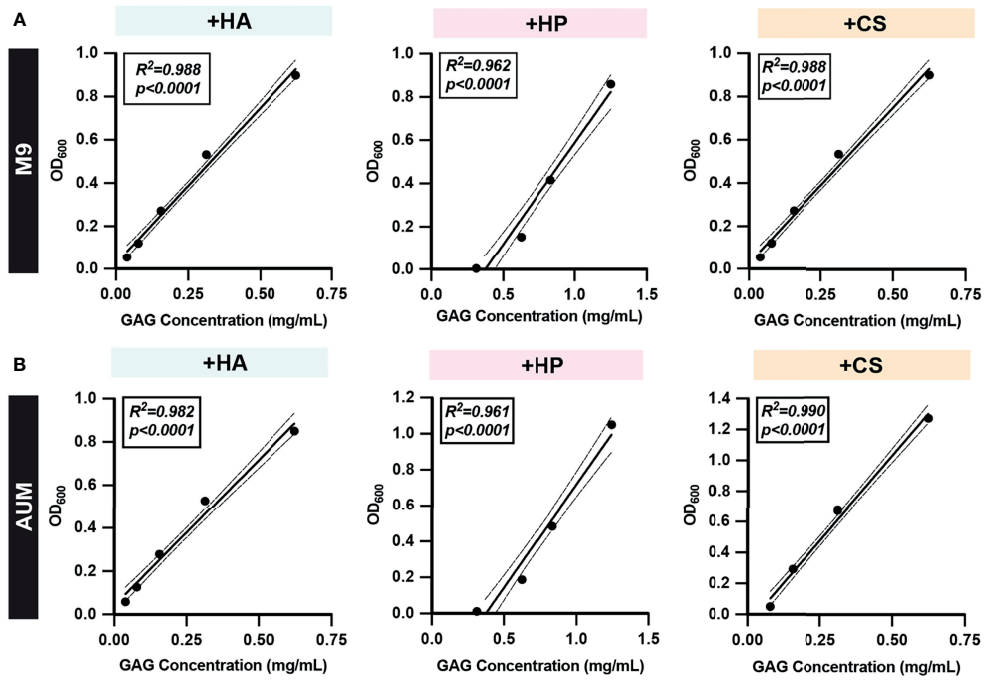

FIGURE 3 | Representative GAG standard curves. Two-fold serial dilutions of **(A)** M9 and **(B)** AUM media with HA, HP, and CS were prepared and the GAG degradation assay protocol was performed to obtain post-acetic acid OD_{600}. Simple linear regressions were performed and R-squared (R^2) and *p*-values are shown. All dots represent the mean across three biological replicates. Solid lines represent line-of-best-fit and dotted lines represent 95% confidence intervals. Values within the linear dynamic range and confidence interval of experimentally generated standard curves were used to calculate semi-quantitative GAG concentrations and interpolate GAG concentrations quantitatively.

E. coli K12 (ECK12), and *S. agalactiae* SA1459, a urine isolate, in the basal media and conditions described in **Table 1** (**Figures 4A–C**). Absolute GAG abundances were estimated by interpolation of the standard curves generated for the respective media type (**Figures 4D–F**). *P. heparinus* was able to completely degrade HA and CS but degraded HP to a lesser extent (**Figure 4**). On the other hand, *S. agalactiae* completely degraded HA, partially degraded CS, and did not degrade HP (**Figure 4**). Lastly, as expected, *E. coli* K12 did not degrade any of the tested GAGs. Importantly, there was agreement between both relative (**Figures 4A–C**) and absolute (**Figures 4D, C**) abundances for each GAG. Overall, these data demonstrate that our developed microtiter plate-based GAG degradation assay can measure degradation of specific GAGs by species previously known to degrade GAGs and is sensitive enough to distinguish between the respective partial and full degradation of CS and HA by *S. agalactiae.* Further, we demonstrated that absolute GAG concentration can be interpolated within the linear dynamic range of the assay.

Optimized Basal and AUM Formulations for Diverse Urinary Bacteria

All GAG degradation and growth assays were performed in a basal media and artificial urine media to mimic the nutrient availability in the urinary tract (**Table S1**). Basal media comprised of an M9 minimal medium was used for *E. coli* and *Klebsiella pneumoniae* strains and supplemented with 3g/L yeast extract ($M9^{Y}$) and/or 10 g/L casamino acids ($M9^{YC}$) for *Enterococcus faecalis*, *Proteus mirabilis*, and *S. agalactiae*. However, a modified MRS (mMRS) media lacking beef extract, dextrose, and sodium acetate was used as a basal media for *Lactobacillus*, *Staphylococcus*, and the other *Streptococcus* species. All *Bifidobacterium* species were tested in mMRS supplemented with 0.5g/L L-cysteine HCl ($mMRS^{L}$). Previously developed AUM formulations were modified with yeast extract (AUM^{Y}), Tween 80 (AUM^{YT}), or L-cysteine HCl (AUM^{YL}) to facilitate the growth of fastidious, Gram-positive urinary bacteria (**Table 1**) (Brooks and Keevil, 1997). All basal and AUM formulations were able to stimulate growth when supplemented with glucose compared to baseline and used as a reference for growth in GAG conditions.

GAG Growth Assay (Endpoint OD_{600})

Our developed semi-quantitative assay measures both GAG degradation and estimates GAG utilization in the same assay. We therefore first used this assay to screen the ability of CS, HP, and HA to stimulate the growth of 39 bacterial strains (37 urinary and 2 control strains). Growth assays were performed to assess microbial growth in the presence or absence of CS, HP, HA and glucose by OD_{600} (**Figures 5** and **S3; Data Set 1**). *E. coli* K12 growth was not stimulated by GAGs but was by glucose (**Figure 5A**). On the other hand, HA and CS significantly

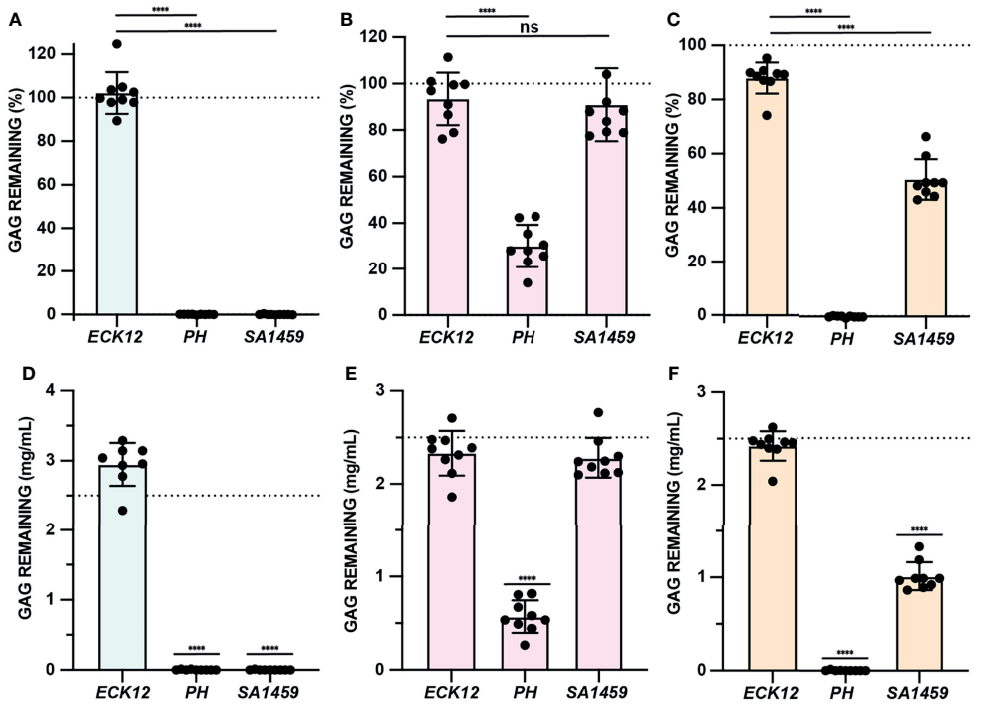

FIGURE 4 | GAG degradation assay validation. **(A–C)** GAG degradation assays were performed and semiquantitative GAG remaining values were shown for ECK12, PH, and SA1459 in basal media supplemented with HA (light blue), HP (light pink), and CS (light tan). An ordinary one-way ANOVA with Dunnett's Multiple Comparisons Test was utilized to compare the GAG activity of negative control ECK12 with PH and SA1459. Dotted line represents a 100% theoretical threshold signifying no GAG degradation. **(D–F)** Absolute abundances of GAGs were interpolated from experimentally generated standard curves and used to assess HA (light blue), HP (light pink), and CS (light tan) degradation in basal media. An ordinary one-way ANOVA with Dunnett's Multiple Comparisons Test was performed to compare interpolated GAG concentrations with a 2.5mg/mL baseline GAG pre-incubation concentration (dotted line). Statistically significant values 1.5 times above baseline are shown (****$p < 0.0001$, ns, not significant).

stimulated endpoint growth in *Pedobacter heparinus*, while HP and glucose did not (**Figure 5B**). This result was in-line with the reduced degradation of HP observed in the validation assay (**Figure 4**). *S. agalactiae* SA1459 showed enhanced growth in HA and glucose, which is consistent with previous studies that found HA can be readily metabolized by *S. agalactiae* (Wang et al., 2014) (**Figure 5C**). Interestingly, growth of *Proteus mirabilis* PM1668 was elevated in $M9^Y$ supplemented with CS compared to $M9^Y$ alone (**Figure 5D**), suggesting that *P. mirabilis* may metabolize CS. Comparatively, growth of *P. mirabilis* in AUM was not significantly stimulated by CS compared to un-supplemented AUM, possibly due the presence of other metabolizable carbon sources in AUM like urea, which plays an important role in *P. mirabilis* virulence (**Figure 5D**) (Armbruster et al., 2018). Although UPEC is the main causative agent of UTI, neither CS, HP, nor HA were able to stimulate growth of UPEC strain EC1318 and other UPEC strains over baseline (**Figure 5E** and **Data Set S1**). GAGs also did not significantly stimulate the growth of *E. faecalis* EF1693, *L. crispatus* LC1700, *L. gasseri* LG637, and *L. rhamnosus* LR1856, in either media type but all grew to higher optical densities in the presence of glucose (**Figures 5F–I**). These results suggest that urinary lactobacilli cannot metabolize HA, HP, and CS in the conditions tested. However, this assay measures GAG utilization, but not GAG degradation. Specifically, it is possible that some bacteria could potentially degrade GAGs to promote host colonization but not utilize it as a source of carbon to stimulate growth.

Semi-Quantitative GAG Degradation Assay

Semi-quantitative GAG degradation assays (**Figure 2**) were performed on 37 urinary isolates, representing 17 species, after endpoint OD_{600} was measured. The results of these assays are presented in **Figures 6**, **S4** and **Data Set 2**. Uropathogenic *E. coli* EC1318, *E. faecalis* EF1693, and *K. pneumoniae* KP1687 did not degrade HA, HP, or CS (**Figures 6A–C**). We did not observe degradation of GAGs by any of the tested UPEC, *E. faecalis*, and *K. pneumoniae* strains (**Data Set S2**). *S. epidermidis* SE730 did not degrade HA, HP, or CS in mMRS or AUM^Y, which is consistent with the previous observation that *S. epidermidis* strain ATCC 12228 does not degrade HA (**Figure 6D**) (Hart et al., 2010). *Bifidobacterium breve* BB158 did not degrade HA, HP, or CS in the conditions tested, which was unexpected because certain fecal strains of *B. breve* have been observed to assimilate both host-derived and dietary glycans (Turroni et al., 2018) (**Figure 6E**). Similarly, *B. longum* BL178 did not degrade HA, HP or CS in the conditions tested (**Figure S4**). Urinary lactobacilli strains *L. jensenii* LJe1708, *L. crispatus* LC1700, and

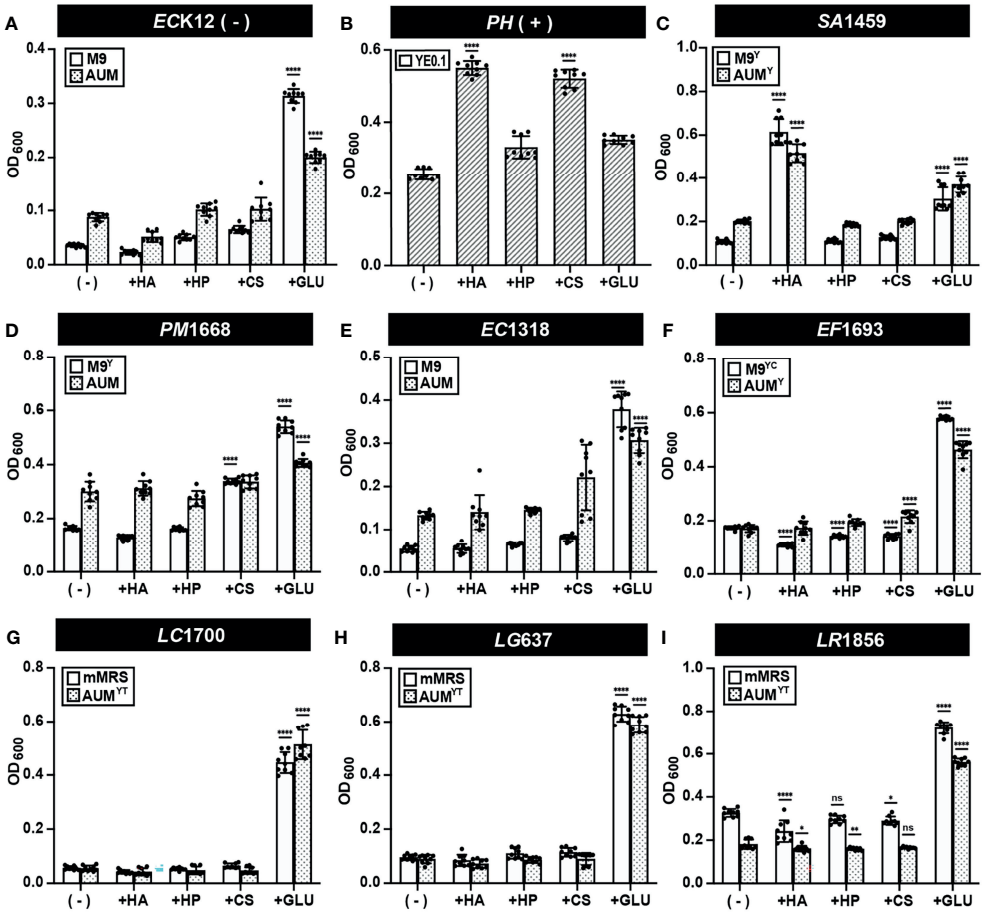

FIGURE 5 | GAG growth assays (Endpoint OD600) of representative strains. Bacterial strains **(A)** *E. coli* K12 **(B)** *P. heparinus* **(C)** *S. agalactiae* SA1459 **(D)** *P. mirabilis* PM1668 **(E)** *E. coli* EC1318 **(F)** *E. faecalis* EF1693 **(G)** *L. crispatus* LC1700 **(H)** *L. gasseri* LG637 **(I)** *L. rhamnosus* LR1856 were cultured in basal or AUM media and supplemented with HA, HP, CS, or glucose and endpoint OD_{600} was measured to assess GAG utilization activity. Assays were performed in three biological replicates and three technical replicates. An ordinary one-way ANOVA with Dunnett's Multiple Comparisons Test was performed to compare basal/AUM media alone (-) and in the presence of GAGs or glucose and statistically significant values 1.5 times above baseline are shown. Dots represent values across three biological replicates and three technical replicates and error bars represent standard deviation (*$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$, ns, not significant).

L. gasseri LG637 did not exhibit any GAG degradation activity in mMRS or AUM^{YT} (**Figures 6F–H**). Although previous work using a qualitative agar-based GAG degradation activity suggested that a fecal isolate of *L. rhamnosus* may degrade HP, we observed neither degradation of HP nor degradation of CS or HA by either of the two urinary *L. rhamnosus* strains tested (Kawai et al., 2018) (**Figure 6I** and **Data Set S2**). Indeed, no tested urinary lactobacilli strain was able to degrade HA, HP or CS in either mMRS or AUM^{YT} (**Figure S4** and **Data Set S2**). Using this method, we observed some values above the 100% GAG remaining threshold and believe that this is an artifact of the assay. However, it is formally possible that these strains could be producing extracellular GAGs, or another molecule that precipitates with BSA and acetic acid. Future work is needed to characterize the biochemical composition of bacterial metabolites produced after incubation with GAGs.

Urinary *Proteus mirabilis* Can Degrade Chondroitin Sulfate

The data from the growth assay suggested that urinary *P. mirabilis* can utilize CS for growth in $M9^{Y}$ (**Figure 5** and **Data Set S1**). We then sought to determine if four urinary *P. mirabilis* strains, PM1668, PM114, PM11, and PM123 were able to degrade CS, HP, or HA *via* our developed GAG degradation assay. The results of the semiquantitative GAG assay show that *P. mirabilis* strains PM1668, PM114, and PM11 degraded CS both in $M9^{Y}$ and AUM (**Figures 7A–C**). The finding of CS degradation in AUM is interesting because CS did not stimulate growth of *P. mirabilis* in AUM (**Figure 5D** and **Data Set S1**). Interestingly, *P. mirabilis* PM123 did not degrade CS in AUM (**Figure 7D**) and *P. mirabilis* PM114 partially degraded HA in $M9^{Y}$. To obtain more quantitative results, we calculated the GAG concentration remaining in the media using standard curves generated in each media type.

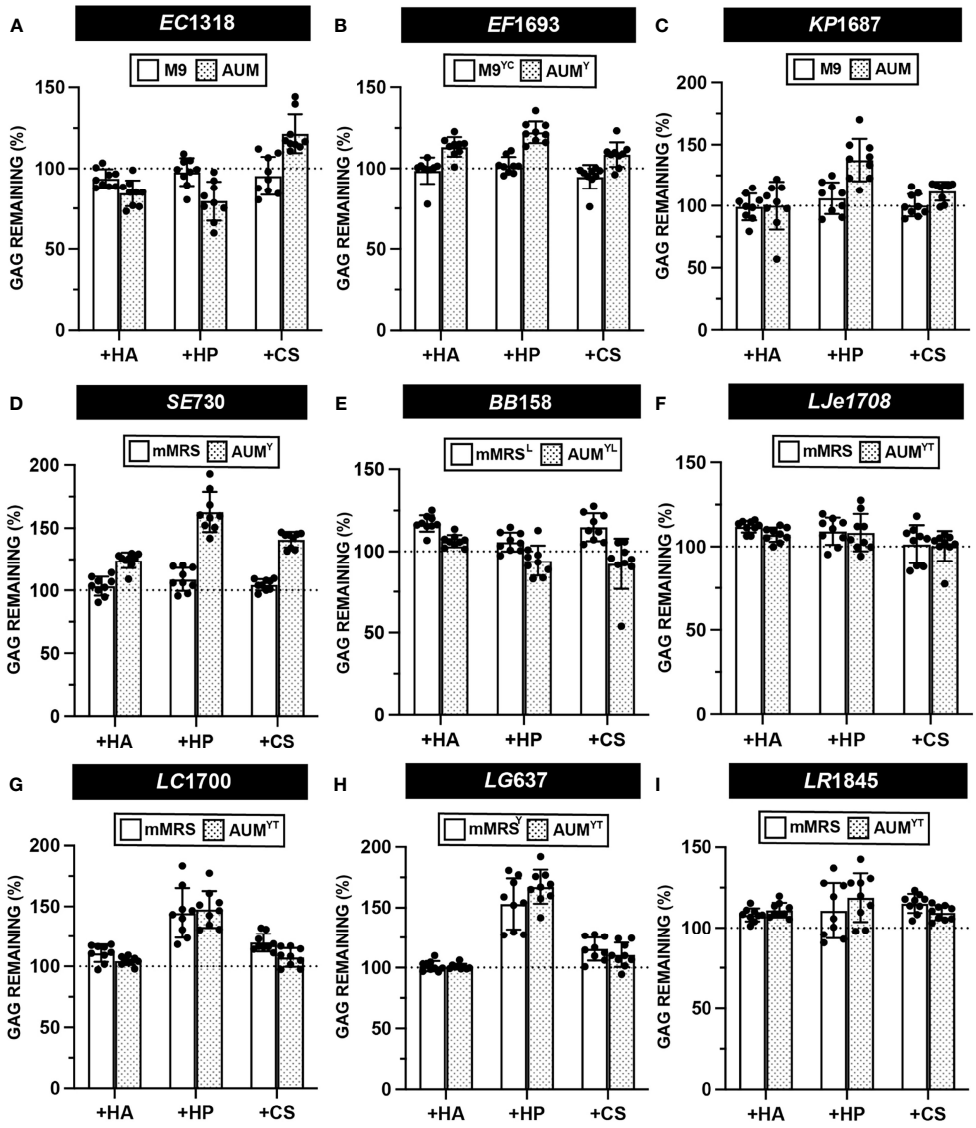

FIGURE 6 | Semi-quantitative GAG degradation by urinary bacteria. GAG degradation assays were performed on strains **(A)** *E. coli* EC1318 **(B)** *E. faecalis* EF1693 **(C)** *K. pneumoniae* KP1687 **(D)** *S. epidermidis* SE730 **(E)** *B. breve* BB158 **(F)** *L. jensenii* LJe1708 **(G)** *L. crispatus* LC1700 **(H)** *L. gasseri* LG637 **(I)** *L. rhamnosus* LR1845 post-incubation with supplemented basal or AUM media and percent GAG remaining was determined using the semi-quantitative formula. Points represent values across three biological replicates and three technical replicates and error bars represent standard deviation from the mean. Dotted line represents a 100% theoretical threshold signifying no GAG degradation.

Similar trends in CS degradation were observed as with the semi-quantitative data (**Figures 7E–H**). Notably, an average of 1.28 mg/mL of HA remained in $M9^{Y}$ following 48 hours of incubation with PM114 (**Figure 7F**). Although the quantitative results were more variable than the semi-quantitative data, on average, 2.27 mg/mL of CS remained in AUM following 48 hours of incubation with PM123 (**Figure 7H**). The unique phenotypes exhibited by PM114 and PM123 suggest some heterogeneity in GAG utilization between *P. mirabilis* strains in different nutrient conditions that warrants future investigation.

DISCUSSION

We developed a semi-quantitative plate-based assay to assess the metabolic potential of 37 urinary microbiota strains to utilize and degrade clinically relevant GAGs in standard basal media and artificial urine media. Our results demonstrate that this assay can capture the well-characterized ability of *S. agalactiae* to degrade HA and partially degrade CS as well as the ability of *P. heparinus* to degrade HA, HP, and CS. We further demonstrate the utility of this assay in screening for GAG degradation activity of diverse

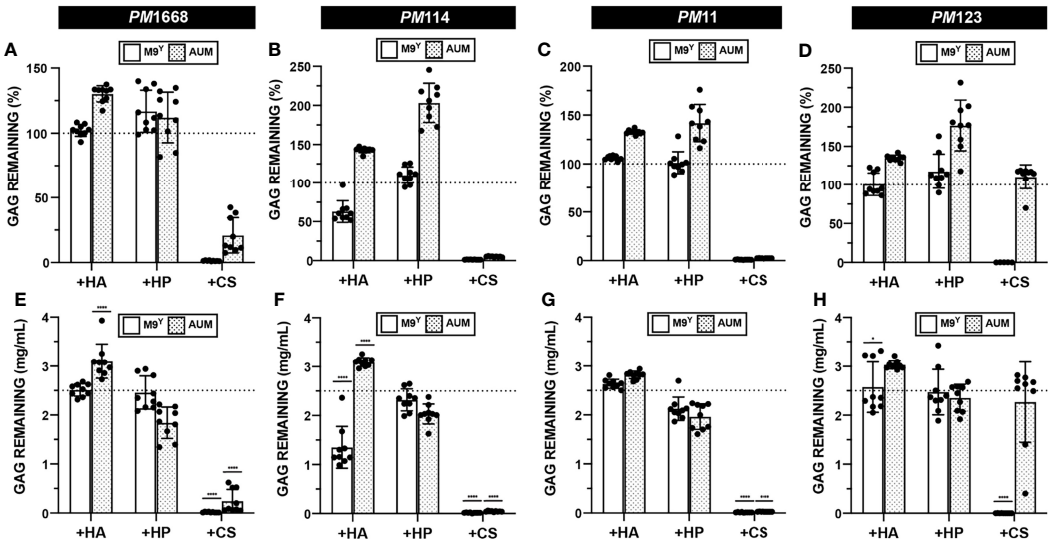

FIGURE 7 | Quantification of GAG degradation by *P. mirabilis*. HA, HP, and CS degradation assays were performed on four separate urinary *P. mirabilis* (PM) strains: **(A, E)** PM1668 **(B, F)** PM114 **(C, G)** PM11 **(D, H)** PM123 in either basal ($M9^Y$) and AUM media. Semi-quantitative GAG percent remaining was calculated **(A–D)**. Absolute abundances of GAGS were interpolated from experimentally generated standard curves **(E–H)**. All points represent values across three biological replicates and three technical replicates and error bars represent standard deviation from the mean. An ordinary one-way ANOVA with Dunnett's Multiple Comparisons Test was performed on absolute abundances to compare interpolated GAG concentrations with a 2.5mg/mL baseline GAG pre-incubation concentration. Statistically significant comparisons with effect sizes >1.25 fold above or below baseline are shown (*$p < 0.05$, ****$p < 0.0001$).

bacterial species. Most importantly, we present the first report of chondroitin sulfate degradation by *Proteus mirabilis*.

This study provides insight into the virulence mechanisms that uropathogenic bacteria may utilize to access the epithelial surface and promote urinary tract colonization. The tested UPEC strains did not utilize or degrade GAGs, which is consistent with previous reports highlighting the importance of amino acid rather than sugar catabolism for UPEC urinary tract fitness (Alteri and Mobley, 2015). On the other hand, degradation of HA and CS by urinary *S. agalactiae* strains may facilitate bladder colonization. *S. agalactiae* hyaluronidase has been previously shown to play a critical role in ascending infection of the female reproductive tract and induction of pre-term birth (Vornhagen et al., 2016). Furthermore, the disaccharides produced during *S. agalactiae* degradation of host HA degradation by hyaluronidase contribute to immune evasion by blocking pro-inflammatory responses mediated by TLR4/2 (Kolar et al., 2015). Likewise, the ability of *P. mirabilis* to degrade CS may contribute to urinary tract colonization, infiltration of the bladder epithelium, immune evasion, or ascending infection. The activity of *P. mirabilis* against CS has not been previously reported, although chondroitinase activity has been reported in *P. vulgaris* (Huang et al., 2003). Our data suggest that *P. mirabilis* can both degrade and utilize CS for growth in $M9^Y$ while only CS degradation was observed in AUM. This phenotype may be due to the ability of *P. mirabilis* to preferentially metabolize other nutrient sources, like urea, present in AUM (Armbruster et al., 2018). Interestingly, *P. mirabilis* PM123 did not degrade CS in AUM, suggesting differences in the regulation of chondroitinase activity between *P. mirabilis* strains. Future genomic analysis to identify and characterize GAGases encoded within the tested *P. mirabilis* strains will help us understand how various culture conditions affect GAGase enzyme expression. These studies will be critical in defining the mechanism and regulation of CS metabolism by *P. mirabilis* and assessing its contribution to bladder colonization or ascending infection.

In addition, we tested species commonly found as part of the urinary microbiota of healthy women, (i.e. *L. crispatus, L. gasseri*, and *L. jensenii*) and observed no GAG utilization or degradation. Although these species may not be able to directly process GAGs, it is formally possible that they may utilize GAG degradation products generated by other members of the urinary microbiota. However, the GAG layer could potentially serve as a scaffolding site for urinary lactobacilli, which are hypothesized to protect against infection similarly to lactobacilli colonizing the vaginal niche (Stapleton, 2016). In an *in vitro* study, GAGs were shown to mediate adherence of *Lactobacillus salivarius* to HeLa cells (Martín et al., 2013). Future investigation should assess the role of the GAG layer as a scaffolding site for urinary lactobacilli at the bladder epithelial surface.

Our novel *in vitro* microtiter plate-based, semi-quantitative GAG degradation and growth assay allows for the rapid screening of GAG degradation and utilization by diverse microbial species in liquid media. An advantage of this method is that it can semi-quantitatively examine differences in GAG degradation activity, whether complete or partial. Compared to qualitative published agar-based assays, this method is higher throughput and is more cost effective because it requires substantially less GAGs per assay (Kawai et al., 2018). Further, this method is an improvement upon existing quantitative assays

like DMMB because it can measure both sulfated and non-sulfated GAGs. Despite these advantages, there are limitations to this method. In the host environment, the microbiota are likely exposed to more than one GAG class and this assay cannot distinguish GAG degradation if more than one GAG is supplemented into the media. Additionally, the GAG degradation products cannot be characterized by this method. Further, because this is an *in vitro* assay, absence of a GAG degradation phenotype does not exclude the possibility that a strain may degrade a given GAG *in vivo*. Expression of GAGases may not be induced under the tested culture conditions, but future genomic analysis will identify and characterize the host and environmental factors that drive the expression of these enzymes. Finally, because this method describes the collection of endpoint growth data, which cannot capture differences in growth rate, it is possible that the addition of GAGs increases or decreases growth rate even in the absence of differences in endpoint growth yields.

In addition to allowing screening of the urinary microbiota, this new method can be easily adapted to enabling screening for GAG degradation and utilization in diverse bacterial species. It can also be adapted to culture conditions (i.e. atmosphere, temperature, media type) representative of diverse biological niches. One obstacle to employing this method to screen other bacterial species is the lack of established minimal media for many understudied bacterial species. Certain members of the urinary microbiota like *Lactobacillus iners*, *Aerococcus urinae, Gardnerella vaginalis, Facklamia* spp. and *Finegoldia* spp. were not screened in this work because minimal media compatible with this assay are still being developed. Also, it is important to note that assay validation would need to be performed to determine the linear range of the assay in new media types. Further assay optimization will allow the screening of additional urinary microbiota species in order to understand the metabolic potential of the microbiota in this important anatomical niche and perhaps the future development of therapies aimed at not only restoring the urinary microbiota but also the integrity of the GAG layer.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Boards of the University of Texas Southwestern Medical Center and the University of Texas at Dallas. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization and method development (VN and ND). Strain curation and maintenance (NH). Experimentation (VN, FK, BS, and NC). Data interpretation and statistical analysis (VN, MN, and ND). Manuscript preparation including writing, figure generation, and editing (VN and ND). Supervision and project management (ND). Funding acquisition (ND and PZ). All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.803409/full#supplementary-material

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ORIGINAL RESEARCH
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A Novel Propidium Monoazide-Based PCR Assay Can Measure Viable Uropathogenic *E. coli In Vitro* and *In Vivo*

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Background: Polymerase chain reaction (PCR) is an important means by which to study the urine microbiome and is emerging as possible alternative to urine cultures to identify pathogens that cause urinary tract infection (UTI). However, PCR is limited by its inability to differentiate DNA originating from viable, metabolically active versus non-viable, inactive bacteria. This drawback has led to concerns that urobiome studies and PCR-based diagnosis of UTI are confounded by the presence of relic DNA from non-viable bacteria in urine. Propidium monoazide (PMA) dye can penetrate cells with compromised cell membranes and covalently bind to DNA, rendering it inaccessible to amplification by PCR. Although PMA has been shown to differentiate between non-viable and viable bacteria in various settings, its effectiveness in urine has not been previously studied. We sought to investigate the ability of PMA to differentiate between viable and non-viable bacteria in urine.

Methods: Varying amounts of viable or non-viable uropathogenic *E. coli* (UTI89) or buffer control were titrated with mouse urine. The samples were centrifuged to collect urine sediment or not centrifuged. Urine samples were incubated with PMA and DNA cross-linked using blue LED light. DNA was isolated and uidA gene-specific PCR was performed. For *in vivo* studies, mice were inoculated with UTI89, followed by ciprofloxacin treatment or no treatment. After the completion of ciprofloxacin treatment, an aliquot of urine was plated on non-selective LB agar and another aliquot was treated with PMA and subjected to uidA-specific PCR.

Results: PMA's efficiency in excluding DNA signal from non-viable bacteria was significantly higher in bacterial samples in phosphate-buffered saline (PBS, dC_T=13.69) versus bacterial samples in unspun urine (dC_T=1.58). This discrepancy was diminished by spinning down urine-based bacterial samples to collect sediment and resuspending it in PBS prior to PMA treatment. In 3 of 5 replicate groups of UTI89-infected mice, no bacteria

grew in culture; however, there was PCR amplification of *E. coli* after PMA treatment in 2 of those 3 groups.

Conclusion: We have successfully developed PMA-based PCR methods for amplifying DNA from live bacteria in urine. Our results suggest that non-PMA bound DNA from live bacteria can be present in urine, even after antibiotic treatment. This indicates that viable but non-culturable *E. coli* can be present following treatment of UTI, and may explain why some patients have persistent symptoms but negative urine cultures following UTI treatment.

Keywords: propidium monoazide, viability, urine, microbiome, non-culturable bacteria, urobiome, urinary tract infection, relic DNA

INTRODUCTION

The existence of the urinary microbiome, the presence of bacterial communities within the urinary tract, is challenging the paradigm that this organ system is normally sterile (Siddiqui et al., 2012; Wolfe et al., 2012; Hilt et al., 2014; Brubaker and Wolfe, 2015; Thomas-White et al., 2016). Furthermore, several studies have shown an association between the urine microbiome and numerous urological diseases (Fouts et al., 2012; Siddiqui et al., 2012; Whiteside et al., 2015; Bajic et al., 2018; Bučević Popović et al., 2018; Magistro and Stief, 2019; Neugent et al., 2020). Therefore, it is imperative to accurately characterize the urinary microbiome as it may inform overall urinary tract health and aid in the diagnosis of urinary conditions, i.e., urinary tract infection (UTI) (Perez-Carrasco et al., 2021).

Numerous clinical studies of patients with UTI feature assessments of both microbiologic and clinical cure, which are based on negative urine cultures and resolution/improvement of symptoms, respectively (Raz et al., 2002; Wunderink et al., 2018; Miller et al., 2019). Some patients in these studies have featured discordance between microbiologic and clinical cure (Hilt et al., 2014; Price et al., 2018; Swamy et al., 2019). One possible interpretation of this discordance is that conventional urine cultures may be missing residual bacteria causing persistent symptoms following antibiotic therapy. An alternative to urine cultures for detection of urinary microorganisms is polymerase chain reaction (PCR). PCR identifies organisms through the amplification of DNA material present in urine and many studies on the urinary microbiome rely on this molecular approach (Lewis et al., 2013; Brubaker and Wolfe, 2015; Ackerman et al., 2019). These methods do not discriminate between relic DNA (DNA from non-viable bacteria) versus DNA from viable bacteria. This is an important limitation of conventional PCR because the confounding effects of relic DNA have been reported in various microbiologic settings (Carini et al., 2016; Nagler et al., 2021; Ren et al., 2021). Viable, metabolically active bacteria presumably exert much more influence over the clinical course of UTI than dead bacteria. Thus, the amplification of total DNA without selection for DNA from viable bacteria may bias conventional PCR-derived results (Carini et al., 2016; Lennon et al., 2018). Given that relic DNA influences conventional molecular measurements of microbial abundance and diversity, we posit that a method to detect viable, metabolically active bacteria is needed for more accurate urobiome studies (Lennon et al., 2018).

A method of identifying metabolically active bacteria *via* PCR has been recently developed. Propidium monoazide (PMA) dye penetrates cells with compromised cell membranes (non-viable cells) and covalently binds to DNA, rendering it unable to be amplified by PCR (Deshmukh et al., 2020). PMA-based PCR has previously been shown to differentiate between non-viable and viable bacteria in many settings (Fittipaldi et al., 2010; Cattani et al., 2016; Gobert et al., 2018; Brauge et al., 2019; Lu et al., 2019). However, the efficiency of PMA in urine has not been previously investigated. Here we investigated the ability of PMA dye in urine to detect DNA derived from viable bacteria.

Abbreviations: CFU, Colony forming units; C_T, Threshold Cycle; dC_T, Delta Threshold Cycle; *E. Coli*, *Escherichia coli;* LB, Luria-Bertani; OD, Optical Density; PBS, Phosphate-buffered saline; PMA, Propidium Monoazide; RT, PCR- Real-time polymerase chain reaction; UTI, Urinary Tract Infection.

MATERIALS AND METHODS

Bacteria Culture

All bacteria work was performed under sterile conditions in a BSL-2 biosafety cabinet. Bacteria were prepared by previously reported methods (Hung, 2009). Briefly, glycerol stock containing the uropathogenic *Escherichia coli* strain UTI89 was used to inoculate a Miller Luria Broth (LB) agar plate (Sigma-Aldrich, St. Louis, MO). The plate was incubated for 24 hours at 37°C. A single colony was picked and transferred to 10 mL LB broth. The culture was incubated overnight at 37°C in a stationary flask. Twenty-five microliters of the 10 mL culture were transferred to 25mL of LB broth. The culture was incubated again overnight at 37°C in a stationary flask. The culture was centrifuged at 5000 x g for 5 minutes at 4°C. The supernatant was decanted and the bacteria pellet was resuspended in 10 mL of sterile phosphate buffered saline (PBS) (Thermofisher Scientific, Waltham, MA). This suspension was diluted tenfold in sterile PBS. The optical density (OD) 600nm value was analyzed using the NanoDrop-1000 (Thermofisher Scientific, Waltham, MA) and the suspension diluted until the OD was 0.50, corresponding to 1-2x10^7 colony-forming units (CFU) per 50 µL. Experiments

involving *Klebsiella pneumoniae* (ATCC reference strain 13883) followed the same procedure described above for UTI89.

Mouse Urine Collection

Mice were scruffed and held with their pelvises above sterile parafilm (Sigma-Aldrich, St. Louis, MO) until they voided. Urine was aspirated from the parafilm. New parafilm was used for each mouse. Urine was then placed on ice and immediately processed.

Clinical Urine Collection

Patient urine was collected according to George Washington University IRB protocol # NCR213442

Generation of Non-Viable Bacteria

Five hundred µL of *E. coli* with OD value 0.5 was mixed with isopropanol (Sigma-Aldrich, St. Louis, MO) to achieve a final concentration of 70% v/v. After 10 minutes, the mixture was centrifuged at 8000 x g for 10 min. The supernatant was removed and the pellet was resuspended in 100 µL of PBS. The suspensions of non-viable bacteria were plated on LB with agar plates and incubated at 37°C overnight to confirm successful killing.

Urine Dilution of Bacteria

Mouse urine was serially diluted to a ratio of 1:2, 1:4, 1:8, and 1:12 with PBS. Subsequently, 50 µL of all viable or all non-viable bacteria was added to 50 µL of the various titrations of urine, or undiluted urine, with resultant ratio of 1:2, 1:4, 1: 8, 1:16, and 1:24. The samples were then either treated with PMA or left untreated.

PMA Treatment

Under minimal light, PMAxx Dye (hereafter referred to as PMA) (Biotium, Fremont, CA) with a concentration of 20 mM was diluted with nuclease-free water (Sigma-Aldrich, St. Louis, MO) to a final concentration of 10 mM. It was then added to the bacterial mixture in a 1:100 ratio. Next, samples were incubated for 15 minutes in the dark with gentle agitation. The samples were placed in an LED lightbox with LED output wavelength of 465-475 nm (Biotium, Fremont, CA) for 20 minutes to induce PMA crosslinking of DNA. The supernatant was removed and the pellet was reconstituted with PBS to its original volume of 100 µL.

DNA Extraction and Quantification

DNA was isolated using the DNeasy PowerSoil Pro Kit (Qiagen, Germantown, MD) according to kit instructions, except that DNA was eluted from the column with 25 µL of nuclease-free water. PCR was performed targeting the *E. coli uidA* gene with TaqMan polymerase (Invitrogen, Waltham, MA) according to previously described methods (Taskin et al., 2011). Delta C_T (dC_T) values were calculated to quantify the differences between C_T values of PMA-treated and untreated samples. For the DNA extraction in the comparison between non-DNA-extracted samples and DNA-extracted samples, 50 µL of cells (0.5-1x10^6 cells) were subjected to DNA extraction, ending in an elution of 50 µL of C6 elution buffer. The PCR quantification was performed in a 25 µL volume in technical triplicates using 2.5 µL of cells (2.5-5x10^5 cells) or 2.5 µL of extracted DNA (which represents the DNA material from 2.5-5x10^5 cells) for each reaction.

Preparation of Urea Solution and PMA Treatment

Urea (Sigma-Aldrich, St. Louis, MO) was diluted with PBS in to two concentrations; 285 mM, corresponding to the urine urea level in humans and 1800 mM, corresponding to the urine urea level in mice (Yang and Bankir, 2005). Fifty µL of 100% viable or 100% non-viable bacteria, corresponding to 1-2x10^7 colony-forming units (CFU) of E. Coli was added to 50 µL of the urea solutions. The DNA of the PMA treated and untreated samples was extracted and amplified as described above.

Titration of Viable and Non-Viable Bacteria With Urine to Develop a Standard Curve

Defined quantities of viable bacteria, isopropanol-killed non-viable bacteria, or PBS were mixed to a total volume of 100 µL. With a fixed amount of viable bacteria (1-2x10^7 CFU), non-viable bacteria were added to achieve 1:10 (1x 10^6 non-viable), 1:100 (1x 10^5 non-viable) and 1:1000 (1x 10^4 non-viable) non-viable to viable bacterial dilutions. In a similar manner, various amounts of viable bacteria (1x 10^6,1 x 10^5, 1x 10^4) were added to a fixed amount of non-viable bacteria (1-2x10^7 CFU) to generate a standard curve. Undiluted viable and non-viable cultures were also used. Fifty µL of these bacterial solutions were added to urine. The mixture was then centrifuged at 5000xg, resuspended with 100 µL of sterile PBS, treated with PMA, and the DNA was extracted as outlined above.

Bacterial Inoculation *Via* Transurethral Catheterization of Mice

All animal work was approved by The Institutional Animal Care and Use Committee of Children's National Hospital under Animal Use Protocol #00030764. Procedures were performed in an ethical fashion. Prior to use, all animals were acclimated for 7 days after arrival to the animal facility. 24-week-old female C3H/HeOuJ mice (stock no: 000635, The Jackson Laboratory, Bar Harbor, ME) were used in this study.

Mice were anesthetized using 2% isoflurane. Any urine in the bladder was expressed by gently pressing on the lower abdomen. A 24g x ¾ inch angiocatheter (Clint Pharmaceuticals, Old Hickory, TN) was attached to a prepared 1 ml syringe containing the inoculant. The angiocath was lubricated (DynaLub Sterile Lubricating Jelly, Amazon, Seattle, WA) and transurethrally inserted into the bladder. 100 µL of the inoculant was instilled slowly into the bladder and the angiocatheter kept inserted for 30 seconds to prevent leakage of the inoculant.

Antibiotic Treatment of Mice

Five days after inoculation, mice were intraperitoneally injected with 10 mg/kg ciprofloxacin twice a day. This regimen was selected as it recapitulates the human plasma peak levels achieved with the commonly used 500 mg oral dose, and has

been shown previously to adequately treat UTI in mice (Guillard et al., 2013).

Urine Collection for *In Vivo* Studies

One day after completion of ciprofloxacin treatment, mouse urine was collected on ice. Individual urine samples in the same treatment groups (3-4 mice/group) were pooled. The urine was either serially diluted and plated in triplicate on LB agar or prepared for PMA treatment. Fifty µL of PBS was added to the urine and the solution was centrifuged and treated with PMA as outlined above. DNA was extracted and the E. coli *uidA* gene was amplified as described above (Taskin et al., 2011).

RESULTS

Urine Interferes With PMA Efficiency

Initial PMA-based PCR experiments using mouse urine spiked with 1-2x10^7 CFU of isopropanol killed UPEC UTI89 yielded little differences in amplification of PMA-treated vs. untreated DNA samples (**Figure 1A**). This led us to consider the possibility that urine was exerting a matrix effect which interferes with downstream molecular processes such as PMA crosslinking (Taylor et al., 2012). The dC_T value of PMA-treated vs. untreated samples that contained 100% non-viable bacteria resuspended in mouse urine was 1.58, which was about one tenth of the dC_T of the same sample resuspended in PBS (13.69). When urine was diluted with PBS, the dC_Ts of PMA-treated vs. untreated samples increased, indicating improved PMA efficiency (**Figure 1B**). However, the increase in dCT plateaued at a dilution of 1:8. These findings indicate that urine inhibits PMA activity.

Urea Does Not Affect PMA Efficacy

Urea is the most abundant solute present in urine and is known to influence molecular structure and function (Yang and Bankir, 2005; Wei et al., 2010). Thus, we sought to investigate urea's potential effect on PMA's function in crosslinking DNA and subsequently inhibiting its amplification. We analyzed PMA's efficiency at two different urea concentrations: 280 mM and 1800 mM, the approximate concentration of urea in human and mouse urine, respectively. The dCTs of PMA-treated vs. untreated samples with 100% nonviable bacteria (1-2x10^7 CFU of E Coli) suspended in either urea concentration was similar to

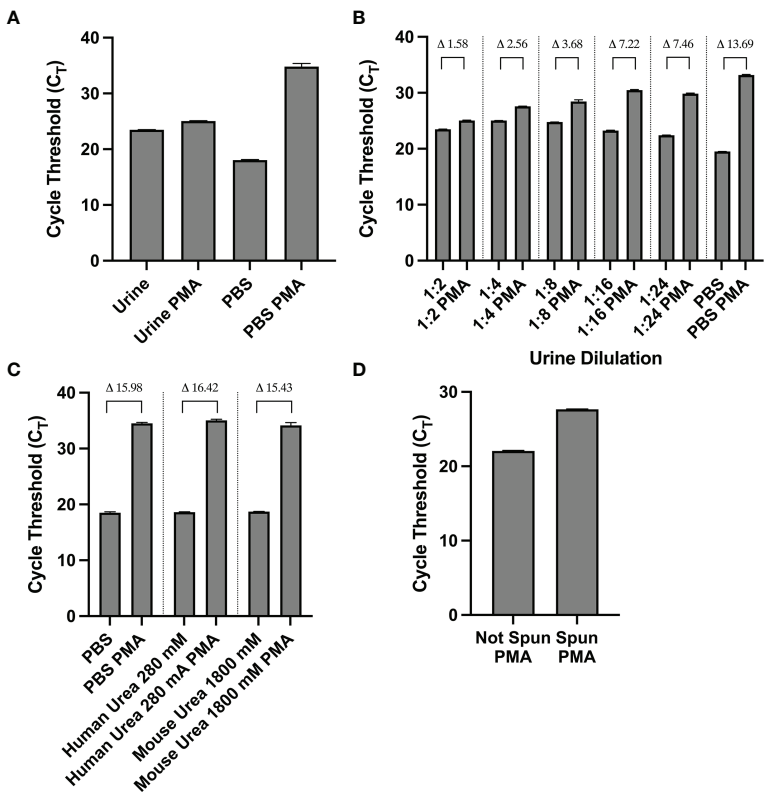


FIGURE 1 | **(A–D)** The matrix effect of urine on PMA crosslinking of DNA is not urea-based and is eliminated by centrifugation and resuspension in PBS. **(A)** C_T values of PMA-treated and untreated samples of non-viable bacteria resuspended in mouse urine or PBS. **(B)** C_T (and indicated dC_T) values of nonviable bacteria resuspended in various dilutions of urine or PBS. **(C)** C_T (and indicated dC_T) values of non-viable bacteria treated with PMA in PBS or urea concentrations corresponding to human and mouse urine. **(D)** C_T values of non-viable bacteria in urine with or without resuspending the contents in PBS before PMA treatment. Data shown is representative of three replicate experiments.

that of the PBS control. Namely, the dCT of the PBS, human urea concentration, and mouse urea concentration samples were 15.98, 16.42, and 15.43, respectively (**Figure 1C**). This suggests that urea does not cause urine's matrix effect on PMA efficiency.

Resuspension of Urine Sediment With PBS Restores PMA Efficiency

We observed that for a solution with 100% nonviable bacteria, the C_T value increases when the urine supernatant was removed and the pellet is resuspended in PBS prior to PMA crosslinking compared to when PMA crosslinking is performed in unspun urine (**Figure 1D**). Furthermore, upon removal of the urine supernatant and subsequent pellet resuspension in PBS, PMA treatment and downstream PCR was most efficient in differentiating viability when there was a greater proportion of nonviable cells in the solution. Across titrations of viable and non-viable bacteria where viable bacteria make up the majority of the solution, C_T values did not significantly differ, with all values close to 16 (**Figure 2A**). This suggests the amount of non-viable bacteria does not influence the detection of a fixed amount of viable bacteria. Conversely, when the majority of the cells are non-viable, the C_T values decrease as the amount of viable bacteria in the solution increases. For instance, the C_T is approximately 18 with 100% viable cells (1-2x10^7 CFU of E Coli) in solution and increases to ~ 27 when viable cells make up only 0.1% of the solution (**Figure 2B**).

Detection of Viable Bacteria Correlates With Colony Forming Units (cfu)

Given that the number of colony forming units present in urine cultures remains the mainstay of clinical diagnosis of UTI, we sought to determine whether urine cultures with various titrations of viable cells and a fixed amount of nonviable cells yielded cfu and C_T values that correlated with each other. Indeed, the correlation between cfu and C_T values was strongly negative with an r^2 of 0.955 (**Figure 3**).

Detection of Non-Culturable But Viable Bacteria in Mouse Urine After Antibiotic Treatment

PCR-based detection of bacterial DNA in urine from patients with persistent UTI symptoms and negative cultures following antibiotic therapy has been criticized as being confounded by the presence of relic DNA (Lehmann et al., 2011). To investigate whether non-culturable, viable bacteria can still be present in urine after antibiotic treatment of UTI, we administered uropathogenic *E. coli* (UTI89) to mice and treated them with ciprofloxacin according to established protocols (Hung, 2009). One day after the completion of antibiotic treatment, 3 out of the 5 replicate groups had no bacterial growth on non-selective LB agar (**Figure 5**). However, after PMA treatment of these samples, PCR successfully amplified the *E. coli uidA* gene in 2 out of the 3 culture-negative groups, indicating the presence of viable, nonculturable bacteria. Based on our standard curve (**Figure 3**), these 2 groups contained 1 and 6×10^5 cfu/ml *E. coli*.

PMA-PCR Can Exclude Signal From Non-Viable *E. coli* in the Presence of Non-Soluble Components of Human Urine, Presence of Non-*E. coli* Bacterial Species, and Omission of DNA Extraction

To further investigate the relevance and feasibility of PMA-based urine PCR for human urine, with considerations for polymicrobial infections and the possibility to decrease the required time and resources for the assay, we spiked culture-negative human urine with different combinations of viable and non-viable *E. coli* and viable *Klebsiella pneumoniae*, followed by PMA treatment and PCR, with or without DNA extraction. We found that, as we observed with mouse urine, in the presence of non-soluble components of human urine

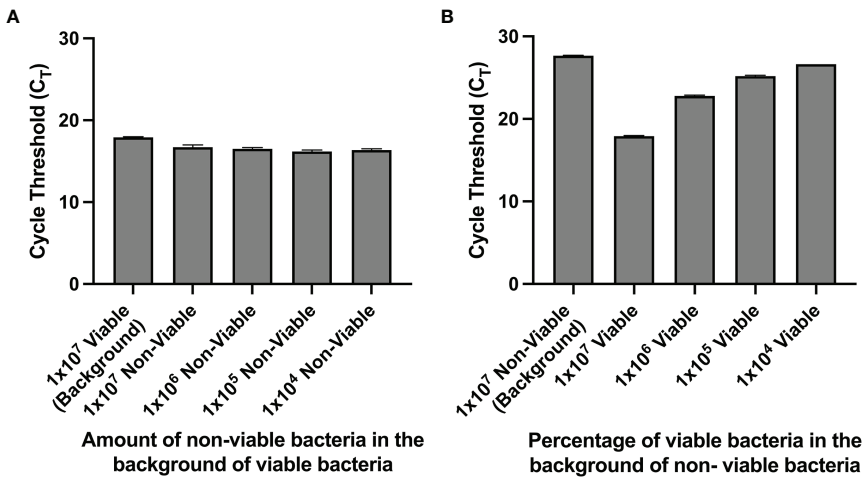


FIGURE 2 | **(A, B)** PMA crosslinking of DNA is most efficient when most of the bacteria is non-viable. Bacterial samples were centrifuged and resuspended in PBS before PMA treatment. **(A)** C_T values of samples with a fixed amount of viable bacteria and titrated amounts of nonviable bacteria. **(B)** C_T values of samples with a fixed amount of non-viable bacteria and titrated amounts of viable bacteria.

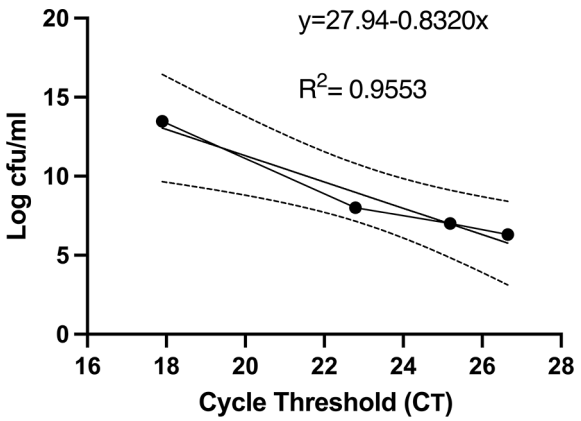


FIGURE 3 | Colony forming units and PMA-based viability PCR cycle threshold number has a strong negative correlation. Before PMA treatment, an aliquot of bacterial samples was serial diluted, plated on LB agar plates, and cfu were counted after 24 hours. cfu per ml were plotted against the respective C_T values. The linear regression and 95% confidence interval band is shown. All experiments were repeated 3 times.

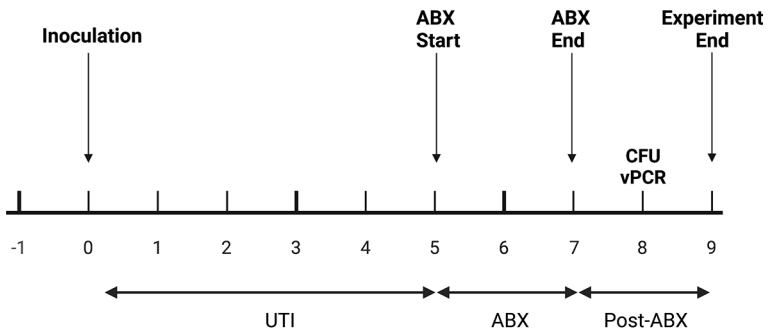


FIGURE 4 | *In vivo experimental plan.* Mice were inoculated with UTI89 *via* transurethral catheterization on day 0 and given ciprofloxacin ("ABX") twice daily on days 5, 6, and 7 post-infection. On day 8, one day after the completion of antibiotics, urine was collected to measure cfu/mL and to perform PMA-based viability PCR ("vPCR").

collected with the bacterial pellet during centrifugation, PMA treatment could exclude signal from non-viable *E. coli* with dC_T values of at least 8.66 (**Figure 6**; samples 1-2, 6-7, 10-11). Similarly, we observed that the presence of *K. pneumoniae*, another commonly found uropathogen, did not strongly affect the signal from *E. coli*, for both PMA-treated and non-PMA-treated samples (**Figure 6**; samples 6-9). Interestingly, DNA-extracted samples showed higher C_T values compared to their respective non-extracted samples, indicating loss of some DNA material during the DNA extraction process; however, this loss of material did not strongly affect PMA function (**Figure 6**; samples 1-4, 6-13).

DISCUSSION

Our findings indicate that a PMA-based urine PCR is an appropriate method to distinguish viable and non-viable *E. coli* in the urine for both *in vitro* and *in vivo* applications. We were able to eliminate the signal from soluble relic DNA and DNA from nonviable cells. By resuspending urine contents in PBS before PMA treatment we established an easy and reproducible method to eliminate soluble relic DNA while preserving *E. coli* cells. This approach yielded dC_T values similar to that of non-urine exposed *E. coli* resuspended in PBS and those reported in the literature (Taskin et al., 2011). Thus, we demonstrate a novel method to utilize PMA in urine that will allow for PCR-based studies to selectively identify viable bacteria in urine.

Our preliminary experiments pointed to a matrix effect of murine urine that inhibited PMA crosslinking of DNA (Chamberlain et al., 2019). We initially focused on urea as a potential cause of this effect. Urea is a by-product of amino acid metabolism and one of the most abundant urine solutes. Mouse urine has a higher urea concentration compared to human urine, which led us to speculate that urea could be influencing PMA crosslinking function in mouse urine (Yang and Bankir, 2005). The similar dC_T of *E. coli* in urea vs. PBS suggested urea is not the substance in urine that inhibits PMA's crosslinking efficiency. Thus, the inhibitory effect of urine is likely due to a non-urea-related

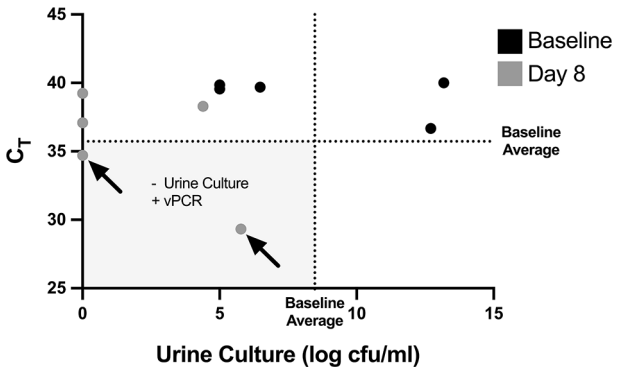
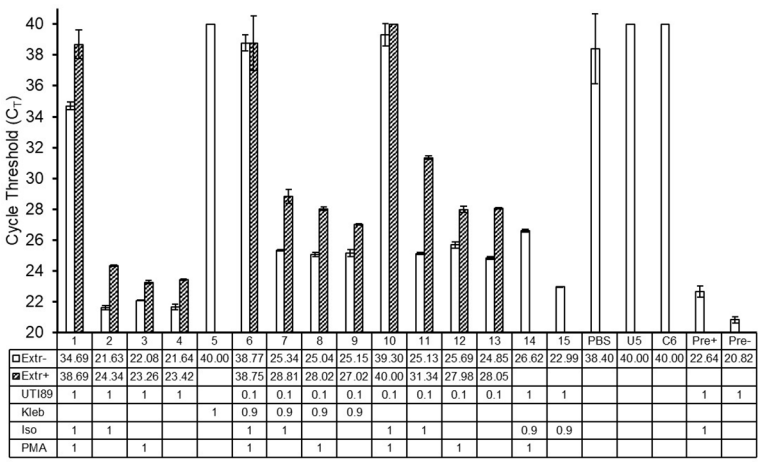


FIGURE 5 | Non-culturable, live bacteria detected in mouse urine by PMA-based PCR after antibiotic treatment. Mice were given UTIs on day 0 and were administered ciprofloxacin starting on day 5 and ending after day 7 (see **Figure 4**). Graph depicts C_T values and log transformed urine cfu/mL values before bacterial inoculation, labeled as baseline (black dots), and at day 8 after antibiotic treatment (grey dots). Each dot represents a pooled cohort of 2-3 mice. The average CT and log cfu/ml of the baseline samples are indicated by the dotted lines. The greyed quadrant (Q3) represents values that are considered as having a negative urine culture and positive PMA-based viability PCR ("vPCR"). Data shown is pooled from two set of experiments.



	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	PBS	U5	C6	Pre+	Pre-
□Extr-	34.69	21.63	22.08	21.64	40.00	38.77	25.34	25.04	25.15	39.30	25.13	25.69	24.85	26.62	22.99	38.40	40.00	40.00	22.64	20.82
▨Extr+	38.69	24.34	23.26	23.42		38.75	28.81	28.02	27.02	40.00	31.34	27.98	28.05							
UTI89	1	1	1	1		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	1	1				1	1
Kleb					1	0.9	0.9	0.9	0.9											
Iso	1	1				1	1			1	1			0.9	0.9				1	
PMA	1		1			1		1		1		1		1						

FIGURE 6 | PMA-PCR can distinguish viable *E. coli* from non-viable *E. coli* in the presence of non-soluble components of human urine, presence of *Klebsiella pneumoniae*, and with or without DNA extraction. Bars represent the arithmetic average of C_T values +/- standard deviation of technical triplicate reactions. Open bars represent samples that did not undergo DNA extraction, while hatched bars represent samples that did undergo DNA extraction. Sample numbers/names and C_T values for non-DNA-extracted and DNA-extracted samples are shown in the table below the graph. PCR samples that did not reach the signal threshold before 40 cycles of PCR, i.e., samples whose C_T could not be determined, were assigned a C_T value of 40. The numbers in the rows labeled "UTI89" and "Kleb" indicate the respective number of bacteria represented in the PCR of each sample, with a value of 1 equating to 2.5-5x10^5 cells. The numbers in the rows labeled "Iso" and "PMA" indicate whether a given sample received the respective treatment, where a value of 1 represents treatment; the value of 0.9 in the "Iso" treatment for samples 14 and 15 indicates that this proportion of cells were treated. In samples 6 and 7, only the UTI89 cells were treated with isopropanol. UTI89, UTI89 strain of *E. coli*; Kleb, *Klebsiella pneumoniae*; Iso, isopropanol; PMA, propidium monoazide; PBS, phosphate buffered saline; U5, culture-negative human urine; C6, DNA extraction elution buffer; Pre+ and Pre-, pre-PMA samples.

matrix effect. The simple steps of centrifuging bacteria-containing samples and resuspending them in buffer may eliminate any matrix effect of other biofluids and environmental samples of interest, enabling PMA-based PCR amplification of DNA from viable bacteria in other settings. While potentially a small number of bacteria can be lost in the supernatant, the standardized centrifuging force and pellet resuspension process used across the various samples should yield similar number of bacteria lost and thus still allow us to perform comparative studies across the samples.

Identifying viable, but potentially unculturable bacteria may improve understanding of bacterial biology in patients with recurrent UTI. We identified non-culturable but viable UTI *E. coli* in the urine of infected mice given ciprofloxacin. Non-culturable but viable bacteria in settings other than the urinary tract are a recognized phenomenon (Coutard et al., 2005; Oliver, 2005). However, the presence of such bacteria in urine is poorly characterized. It may be that these bacteria represent intracellular UTI *E. coli* which have formed intracellular communities and

become quiescent reservoirs of infection (Mulvey et al., 2001; Rosen et al., 2007). Our findings may explain why, despite patients having undergone susceptibility-guided antibiotic treatment and a subsequent negative test-of-cure by urine culture, some of these patients experience recurrent UTI.

Compared to urine culture, PMA-based urine PCR has the clinical advantages of a more rapid time to UTI diagnosis and broader organism detection. While enhanced quantitative urine culture has demonstrated greater sensitivity for uropathogen detection than conventional culture (Price et al., 2016), it is still time-intensive. In contrast, a uropathogen-specific PCR platform based on PMA could detect multiple viable organisms quickly.

This is a preliminary study using a new molecular method for identification of bacteria in urine. A potential limitation to our study is the use of PMA dye prior to PCR. Specifically, studies have shown that PMA results can be skewed by specific primers. However, the primers used in this study have been shown to be effective in multiple studies for *E. coli* without loss of viability data (Taskin et al., 2011; van Frankenhuyzen et al., 2013).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Children's National and the Children's National Research Institute-Institutional Animal Care and Use Committee. The human urine study protocol was approved by George Washington University Institutional Review Board.

AUTHOR CONTRIBUTIONS

AL, OL, and MH contributed to conception and design of the study. AL and OL organized the database. OL performed the statistical analysis. AL and OL wrote the first draft of the manuscript. KI, EH, AN, and MH wrote sections of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Network-Based Differences in the Vaginal and Bladder Microbial Communities Between Women With and Without Urgency Urinary Incontinence

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Background: Little is known about the relationship of proximal urogenital microbiomes in the bladder and the vagina and how this contributes to bladder health. In this study, we use a microbial ecology and network framework to understand the dynamics of interactions/co-occurrences of bacteria in the bladder and vagina in women with and without urgency urinary incontinence (UUI).

Methods: We collected vaginal swabs and catheterized urine specimens from 20 women with UUI (cases) and 30 women without UUI (controls). We sequenced the V4 region of the bacterial 16S rRNA gene and evaluated using alpha and beta diversity metrics. We used microbial network analysis to detect interactions in the microbiome and the betweenness centrality measure to identify central bacteria in the microbial network. Bacteria exhibiting maximum betweenness centrality are considered central to the microbe-wide networks and likely maintain the overall microbial network structure.

Results: There were no significant differences in the vaginal or bladder microbiomes between cases and controls using alpha and beta diversity. Silhouette metric analysis identified two distinct microbiome clusters in both the bladder and vagina. One cluster was dominated by *Lactobacillus* genus while the other was more diverse. Network-based analyses demonstrated that vaginal and bladder microbial networks were different between cases and controls. In the vagina, there were similar numbers of genera and subgroup clusters in each network for cases and controls. However, cases tend to have more unique bacterial co-occurrences. While *Bacteroides* and *Lactobacillus* were the central bacteria with the highest betweenness centrality in controls, *Aerococcus* had the highest centrality in cases and correlated with bacteria commonly associated with bacterial vaginosis. In the bladder, cases have less than half as many network clusters compared to controls. *Lactobacillus* was the central bacteria in both groups but

associated with several known uropathogens in cases. The number of shared bacterial genera between the bladder and the vagina differed between cases and controls, with cases having larger overlap (43%) compared to controls (29%).

Conclusion: Our study shows overlaps in microbial communities of bladder and vagina, with higher overlap in cases. We also identified differences in the bacteria that are central to the overall community structure.

Keywords: urobiome, urinary microbiome, vaginal microbiome, urgency urinary incontinence, network analysis

INTRODUCTION

Urgency urinary incontinence (UUI), which is defined as involuntary urinary leakage accompanied by or immediately preceded by urgency (Haylen et al., 2010), affects up to 30% of women with increasing risk with age (Coyne et al., 2012). Women are twice as likely to be affected by UUI as men. Many etiologies have been proposed for UUI ranging from neurogenic to idiopathic (less understood) causes. The underlying pathophysiologic factors that contribute to non-neurogenic UUI are thought to range from abnormal sensory function at the level of the urothelium or urethra (de Groat, 1997; Yamaguchi et al., 2007) to involuntary myogenic (detrusor) contractions (Abrams et al., 2002), altered brain function (Griffiths et al., 2009; Nardos et al., 2014; Nardos et al., 2016), and more recently, a shift in urinary bladder microbiome (Brubaker et al., 2014; Pearce et al., 2014; Pearce et al., 2015; Karstens et al., 2016). Other factors such as metabolic disorders (Bunn et al., 2015), affective disorders (Vrijens et al., 2015) and hormonal changes (Cody et al., 2012) have also been shown to be associated with this condition. These pathophysiologic factors are likely not mutually exclusive.

Recent advances in both culture and culture-independent techniques have made it possible to evaluate the role of microorganisms more thoroughly in health and disease. With these breakthroughs came the discovery that microbes contribute to human health more extensively than initially thought (Haiser et al., 2013; Fulde and Hornef, 2014; Neuman et al., 2015). Recent studies demonstrate that resident bacteria in the bladder may have a role in healthy bladder function (Siddiqui et al., 2011; Fouts et al., 2012; Wolfe et al., 2012; Khasriya et al., 2013; Lewis et al., 2013; Hilt et al., 2014; Wolfe and Brubaker, 2015). An alteration in the resident bladder bacterial community, on the other hand, may be associated with bladder disorders such as overactive bladder (OAB) with or without UUI (Brubaker et al., 2014; Pearce et al., 2014; Pearce et al., 2015; Karstens et al., 2016; Drake et al., 2017; Aragón et al., 2018) and interstitial cystitis (Siddiqui et al., 2012).

It is generally understood that the gut is the main source of urinary uropathogens by way of an intermediary, i.e., vagina. The female urogenital tract is particularly amenable to this migration theory given the anatomical characteristics and proximity of these organs to each other. The vaginal microbiome is therefore thought to play a critical role in contributing to bladder health in addition to its well-established role in maintaining a healthy vaginal environment (Ravel et al., 2011; Greenbaum et al., 2019). However, little is known about how these proximal ecosystems, i.e., microbiomes in bladder and vagina, relate to each other to affect bladder health or disease. In this study, we use a microbial network framework to understand the shift in the dynamics of interactions or co-occurrences of bacteria in the vagina and urinary bladder in women with and without UUI. Such application of network analysis methods allow us to understand the larger microbial community structures of the bladder and vagina and how they differ in the urogenital tract of women with and without UUI.

MATERIALS AND METHODS

This observational case–control study was conducted at the Oregon Health & Science University (OHSU) between 2016 and 2019. Study approval was obtained from the OHSU's Institutional Review Board (IRB 00010729) as part of a larger effort to understand the role of urinary bladder microbiome in overactive bladder syndrome in women. Women between the ages of 45 and 85 were recruited both from the general population in the Portland area and through the Pacific Northwest Pelvic Floor Research Group, urogynecology clinical providers and researchers from the OHSU, Kaiser Permanente NW, and affiliated Portland-area hospitals. Participants were prescreened over the phone and those who met inclusion criteria completed their study visits at the OHSU's Women's Health Research Unit (WHRU).

We recruited twenty women with UUI (cases) and thirty women with normal bladder function (controls). The UUI group included women with daily urge-predominant incontinence confirmed on a three-day voiding diary, with urge-predominant leakage as determined by a Patient Perception of Intensity Urgency Scale (PPIUS) (Cartwright et al., 2010) score ≥3 (severe urgency that I could not postpone voiding) for >50% of total incontinence episodes on diary. The control group included female participants without a history of any UUI symptoms or frequent (>once a week) stress incontinence symptoms based on screening questionnaire and confirmed on three-day voiding diary. Participants were excluded if they had urinary retention with a baseline need for intermittent self-catheterization, known neurological diseases that could affect bladder function (stroke, multiple sclerosis, brain or spinal cord injury, myasthenia gravis), current pregnancy or lactation, history of pelvic radiation, current

pelvic or bladder malignancy, symptomatic urinary tract infection detected on screening urinalysis and confirmed with culture (growth of $>10^5$ colonies per ml), symptomatic pelvic organ prolapse (sensation of vaginal bulge), prior or current diagnosis of painful bladder syndrome, or a history of antibiotics in the previous two months.

All participants provided written consent and completed a demographic and health questionnaire and a three-day bladder diary. Participants were asked to score their urinary urgency on the bladder diary using the PPIUS. Participants also completed the International Consultation on Incontinence Questionnaire (ICIQ) (Avery et al., 2004), the Pelvic Floor Distress Inventory Urogenital Distress Inventory (UDI) (Barber et al., 2005) and the Overactive Bladder Questionnaire (OAB-q) (Coyne et al., 2002). These validated questionnaires assess urinary incontinence symptoms, impact of pelvic floor disorders on daily function, quality of life, symptom bother, and health-related quality of life, respectively. Finally, participants completed the Patient Global Impression of Severity (PGI-S) (Yalcin and Bump, 2003), the Patient Perception of Bladder Condition (PPBC) (Coyne et al., 2006) and the Beck's Anxiety Inventory (Fydrich et al., 1992). During their study visit, urine was collected from the bladder using an aseptic technique with a urethral catheter by a trained and licensed practitioner. The total volume of urine was emptied, and urine specimens were aliquoted into sterile 50 ml conical tubes and stored at −20°C until further processing. Mid-vaginal swabs were also collected by a trained and licensed practitioner from the same participants on the same study visit. This was done by inserting a sterile cotton-tipped swab into the vagina, rotating the swab 360° five times, and letting the swab sit in mid vagina for 20 s. All specimens were handled in a sterile biosafety cabinet after collection.

Statistical Analyses

Differences in clinical and demographic characteristics between UUI and controls were assessed with Student's t-tests for normal and continuous characteristics, Kruskal–Wallis' rank sum test for non-normal and continuous characteristics, and Fisher's exact test for categorical data. The Shapiro–Wilk test was used to test for normality prior to testing. Clinical covariates that were found to be statistically different between UUI cases and controls were considered as covariates in downstream analyses. Data management, descriptive statistics, visualizations, and analyses were performed in R (version 3.6.1) (Team, 2017).

Molecular Methods

Microbial DNA from vaginal swabs was extracted by vortexing swab heads in PowerBead tubes before centrifugation at 10,000*g* for 30 s at room temperature following the DNeasy PowerSoil DNA isolation kit (QIAGEN, Germany). Microbial DNA from the urinary bladder was extracted from microbial pellets formed from the centrifugation of 20–45 ml of urine at 10,000*g* for 30 min twice. DNA extraction was performed using the cultured cells protocol supplied with the DNeasy Blood and Tissue Kit (QIAGEN, Germany). The extracted DNA was quantified and quality checked at A260/A280 nm (Nanodrop, Thermo Fisher Scientific, USA) prior to amplification by polymerase chain reaction (PCR). No template controls and a mock microbial dilution series were also extracted with each protocol and subjected to amplification and sequencing.

Bacterial DNA was amplified by PCR using Golay barcoded primers which target the V4 region of 16S rRNA genes (Caporaso et al., 2012). Template DNA was amplified in triplicate using the GoTaq Hot Start Polymerase kit (Promega, USA). One microliter of template DNA and 1 µl of a unique barcoded reverse primer were added to 48 µl of master mix containing 1× colorless reaction buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 mM forward primer, and 1.25 U of polymerase enzyme. The reaction volumes were placed in a thermocycler and run through the following conditions: 94°C for 3 min (initial denaturation), followed by 35 cycles of 94°C for 45 s (denaturation); 55°C, 40 s (annealing); 72°C, 1.5 min (extension); with a final extension at 72°C for 10 min.

Ten microliters of each product were used to verify amplification by gel electrophoresis on a 2% agarose gel. Replicates yielding visible bands at 382 bp were pooled together and purified following the QIAquick PCR Purification kits (QIAGEN, Germany) provided protocol. Purified products were again quantified, and quality checked at A260/A280 nm (Nanodrop, Thermo Fisher Scientific, USA). Products were diluted to 10 ng/µl, and 5 µl of each sample were pooled together for sequencing on the Illumina MiSeq sequencer (Illumina, USA).

Sequence Processing

Illumina sequence reads were processed using DADA2 (version 1.4.0) (Callahan et al., 2016) to yield amplicon sequence variants (ASVs), using default parameters unless otherwise noted. Briefly, reads were trimmed 10 bases from the 5' end for both forward and reverse reads, and the 3' ends were truncated to 240 and 160 bases, respectively. Chimeric sequences were identified and removed by taking a consensus across samples using the removeBimeraDenovo function. Taxonomy was assigned using the RDP classifier (Wang et al., 2007) with the Silva database (version 132) as implemented in the assignTaxonomy function. For further manipulations, we used Phyloseq (version 1.28.0) (McMurdie and Holmes, 2013) and several other R packages. ASVs were agglomerated to the genus taxonomic rank for downstream analyses. For diversity analyses, vaginal and bladder microbiome sequence variants were rarefied without replacement to 15,000 reads per sample and 2,500 reads per sample, respectively. Performed separately for vaginal and urine samples, genera that contributed greater than 5% of the total of at least one sample were considered for further analysis. Identification and removal of contaminant sequences was performed on urinary bladder microbiome samples using the Decontam (version 1.4.0) (Davis et al., 2018), using the frequency classification with a threshold of 0.3. Decontam was also performed on vaginal samples using the threshold of 0.5. Phylogenetic trees were constructed by generating a neighbor-joining tree based on a multiple sequence alignment as implemented with default parameters in DECIPHER (version 2.14.0) (Wright, 2016) and Phangorn (version 2.5.5) (Schliep, 2011).

Microbiome Analyses

Stacked bar plots based on sequence abundance were produced for the vaginal and bladder microbiome samples using Microshades (Dahl et al., 2021). Weighted UniFrac distance was calculated between samples, and the updated Ward's minimum variance method was used for agglomerative hierarchical clustering (Murtagh and Legendre, 2014) with complete linkage using hclust "ward.D2". The clustering dendrogram was cut based on the silhouette clustering metric, a measure assessing the similarity of within-cluster points with other cluster points. The silhouette clustering metric was calculated for potential clusters of 2 through 6, and the largest value was used as the optimal number of clusters. A dendrogram was used to visualize the hierarchical clustering relationships *via* the Dendextend (version 1.13.4) (Galili, 2015).

Alpha and beta diversity were calculated for the UUI case and control samples using Phyloseq (McMurdie and Holmes, 2013), Vegan (version 2.5.6) (Oksanen et al., 2019), and Microbiome (version 1.6.0) (Lahti and Shetty, 2017) R packages. Alpha diversity was assessed with the observed number of taxa, Pielou's evenness index (Pielou et al., 2007), and inverse Simpson index. Beta diversity between subject samples was calculated using the Bray-Curtis, weighted UniFrac and unweighted UniFrac (Lozupone and Knight, 2005) distance measures using the distance function in the Phyloseq, visualized using principal coordinates analysis (PCoA), and assessed with PERMANOVA (Anderson, 2014) for significance using the adonis function in the Vegan package.

Network Analyses

SparCC was used to construct microbial networks. SparCC accounts for the compositional nature of 16S rRNA data by performing a linear Pearson correlation on log-ratio transformed data (Friedman et al., 2004). This transformation is beneficial because it retains the true abundance values as a ratio, which are independent of other taxa included in the data, and the transformation can take any value rather than being constrained to a fixed abundance. The SparCC method was performed as implemented in the sparcc function with default parameters in the SpiecEasi package (version 1.0.7) (Kurtz et al., 2015). Network analyses were performed using the R packages tidygraph (version 1.2.0) (Pedersen, 2020), with the underlying functionality of igraph (version 1.2.5) (Csardi and Nepusz, 2006), and visualized using the R package ggraph (version 2.0.3) (Pedersen, 2021). Community detection was performed using the InfoMap community detection algorithm, which minimizes the expected description length of a random walker along the network, as implemented in cluster infomap in the R package igraph (Rosvall and Bergstrom, 2008).

A permutation analysis was used on all UUI case and control vaginal microbiome data to determine a correlation threshold by shuffling the sample labels for each genus in a pairwise comparison prior to calculating SparCC correlations. A similar permutation was performed separately on the bladder microbiome data. This permutation analysis generates a null distribution of correlations from which to identify a threshold of correlations for downstream analyses. A permutation of 1,000 trials was performed and a threshold of the top 5% of the null distribution was used to determine a correlation cut off for each the vaginal microbiome (correlations >0.23) and bladder microbiome data (correlations >0.22). Only positive correlations were considered for the network analysis.

RESULTS

The study included twenty women with UUI and thirty women without UUI (controls). Women in both groups were similar in age, menopause status, estrogen use, number of vaginal deliveries, and race ($p > 0.05$) (**Table 1**). There was also no significant difference in clinical history such as IBS, history of pelvic surgery, diabetes, or current tobacco use. However, women with UUI were more likely to have had a history of recurrent UTIs compared to controls ($p = 0.007$). Women with UUI were also significantly more likely to score higher on Beck's Anxiety Inventory (10.6 ± 12.0) compared to controls (2.6 ± 2.6) ($p < 0.001$) even though they did not report a higher incidence of anxiety diagnosis in their medical history.

As expected, the UUI group had higher scores on clinical symptom and symptom bother questionnaires related to incontinence (UDI, OAB-q, ICIQ, $p < 0.001$, **Table 2**). The higher UDI-6, OAB-q symptom bother, and ICIQ scores for the UUI cohort indicate the severity of symptom bother and disability in this particular population. Similarly, the lower OAB-q health-related quality of life scores for the UUI cohort indicates a lower quality of life in the UUI population. Objective measures of incontinence are captured by a three-day bladder diary which showed that 60% of the UUI population had daily urge leaks and 15% had daily stress leaks while the control group did not have any urge or stress leaks. There was no significant difference in nocturia or daytime urinary frequency between the two groups.

The 16S rRNA amplicon sequencing resulted in a mean sequencing read depth of 45,005 reads per sample (range 15,201–69,787) for vaginal samples and 38,941 reads per sample (range 2,319–137,986) for urine samples. There were no significant differences in the number of reads per sample between the UUI and control groups ($p = 0.7$ vaginal samples, $p = 0.2$ urine samples). After processing and filtering as described in the methods, the vaginal sequencing resulted in classification of 8 phyla, 12 classes, 18 orders, 31 families, and 66 genera. Urine sequencing resulted in classification of 14 phyla, 21 classes, 43 orders, 70 families, and 131 genera.

Vaginal Microbiome

We identified two distinct clusters of vaginal microbiome profiles (silhouette score 0.63, **Figure 1A**). One cluster was dominated by the genus *Lactobacillus*, while the second cluster included microbiomes that were dominated by *Gardnerella*, *Bifidobacterium*, *Escherichia* or had no dominant bacteria and contained a mixture of *Anaerococcus*, *Prevotella*, *Escherichia*, *Gardnerella*, *Bifidobacterium*, or other genera. We tested the relationship between these vaginal microbiome clusters and demographic/clinical characteristics and found no association with cohort status (UUI vs. control) ($p = 1.0$) or menopausal status ($p = 1.0$). However, the clustering was associated with vaginal product use,

TABLE 1 | Participant demographics and comorbidities.

	UUI (N = 20)	Control (N = 30)	p-value
Age (years)	64.2 ± 10.5	57.9 ± 10.4	0.04
Body mass index (kg/m^2)	29.25 [25.93, 32.8]	25.4 [23.2, 28.3]	0.005
Menopause status			0.32
Premenopausal	3 (15%)	9 (30%)	
Postmenopausal	17 (85%)	21 (70%)	
Any Estrogen use	9 (45%)	7 (23%)	0.22
Race			0.68
White	18 (90%)	27 (90%)	
Non White	2 (10%)	3 (10%)	
Vaginal delivery (Yes)	11 (55%)	17 (47%)	1.00
Number of vaginal deliveries	2.00 [1.50, 3.00]	2.00 [1.00, 3.00]	0.24
History of diabetes	4 (20%)	6 (7%)	0.14
Smoking (current)	1 (5%)	0 (0%)	0.40
Has history of recurrent UTI	5 (25%)	0 (0%)	0.007
History of Anxiety	4 (20%)	4 (13%)	0.27
Beck's Anxiety Inventory Score	7.50 (2.5, 15.25)	2.0 (1.00, 3.00)	0.007
History of IBS	4 (20%)	2 (10%)	0.28
History of Pelvic Floor Surgery	7 (35%)	11 (37%)	1.00

Student's t-test was performed on continuous, normally distributed data and displayed with mean and standard deviation. The Kruskal–Wallis test was performed on continuous, non-normally distributed data and displayed with median and the interquartile range. The Fisher's Exact test was performed on categorical data and counts reported as number of individuals with corresponding demographic or condition.

with more participants in the *Lactobacillus*-dominated clusters reporting use of vaginal products (p = 0.004). Vaginal products were defined as any use of vaginal medication or suppository, douche, feminine spray, spermicide, or personal lubricant in the week prior to collection of vaginal microbiome samples. There was no difference in either alpha diversity (**Figure 1B**) or beta diversity in the vaginal microbiome of women with and without UUI (**Supplemental Figure 1** PERMANOVA p = 0.71, adjusted for age, BMI, menopause status, and estrogen use). Of the 16 participants who reported use of vaginal products, 13 reported vaginal estrogen use as the vaginal product. Therefore, we excluded vaginal product from the adjusted model and only included estrogen use.

For each group, we inferred a microbiome-wide interaction network based on the bacterial genera in the vaginal microbiome (**Figure 2** and **Table 3**). Of note, interaction in this correlation-based network does not indicate physical or biochemical interactions among microbes. There was a similar number of genera in the microbiome networks of both UUI and control groups (55 genera versus 52 genera respectively) but more unique bacterial co-occurrences (pairs of bacteria that co-occur) in the UUI group compared to controls (343 associations versus 152 associations respectively). The number of clusters in the vaginal network were similar between UUI and controls, where the UUI network clustered into 5 subgroups of bacteria and controls clustered into 6 subgroups.

We further explored the presence of a central bacteria in each of the vaginal networks using the betweenness centrality measure (**Table 4**). Central bacteria have high betweenness centrality, i.e., in the path between most other bacterial networks, potentially leveraging higher influence over network stability. *Bacteroides* were the central bacteria in the vaginal microbiome-wide network of control samples with the highest centrality measure of 0.39, followed by *Lactobacillus* (betweenness centrality of 0.19). In the UUI group, *Aeroccocus* were the central genus in the vaginal microbiome-wide network with a centrality measure of 0.33, followed by *Streptococcus* (betweenness centrality of 0.22). *Lactobacillus* were not found to be in the top six most central bacteria in the vaginal microbiome-wide network of UUI subjects. We further explored the surrounding bacterial associations to the central bacteria in the UUI group (**Table 5**). These top associated bacteria include *Actinomyces*, *Staphylococcus*, *Helcococcus*, *Streptobacillus*, *Prevotellaceae*, *Gardnerella*, and *Bacteroides*.

Bladder Microbiome

For the bladder microbiome, we identified two distinct clusters using the silhouette metric which is similar to vaginal microbiome (highest silhouette score of 0.50, **Figure 3A**). One cluster had a diverse mix of bacteria, namely, *Bacteroides*,

TABLE 2 | Participant bladder symptoms.

	UUI	Control	p-value
Urogenital distress inventory (UDI-6 Short Form)	5.5 [4.8, 9.0]	0.0 [0.0, 0.0]	<0.001
Overactive Bladder Questionnaire (OAB-q) symptom bother	45.0 [40.0, 70.0]	6.7 [3.3, 15.0]	<0.001
OAB-q health-related quality of life	66.9 [45.8, 78.9]	98.5 [95.8, 100.0]	<0.001
International Consultation on Incontinence Questionnaire (ICIQ)	10.5 [8.0, 14.3]	3.0 [0.0, 3.0]	<0.001

This table summarizes bladder symptoms, assessed by validated pelvic floor questionnaires. Statistics performed by Kruskal–Wallis, comparing UUI cases to controls and displayed with the median and IQR. IQR, interquartile range.

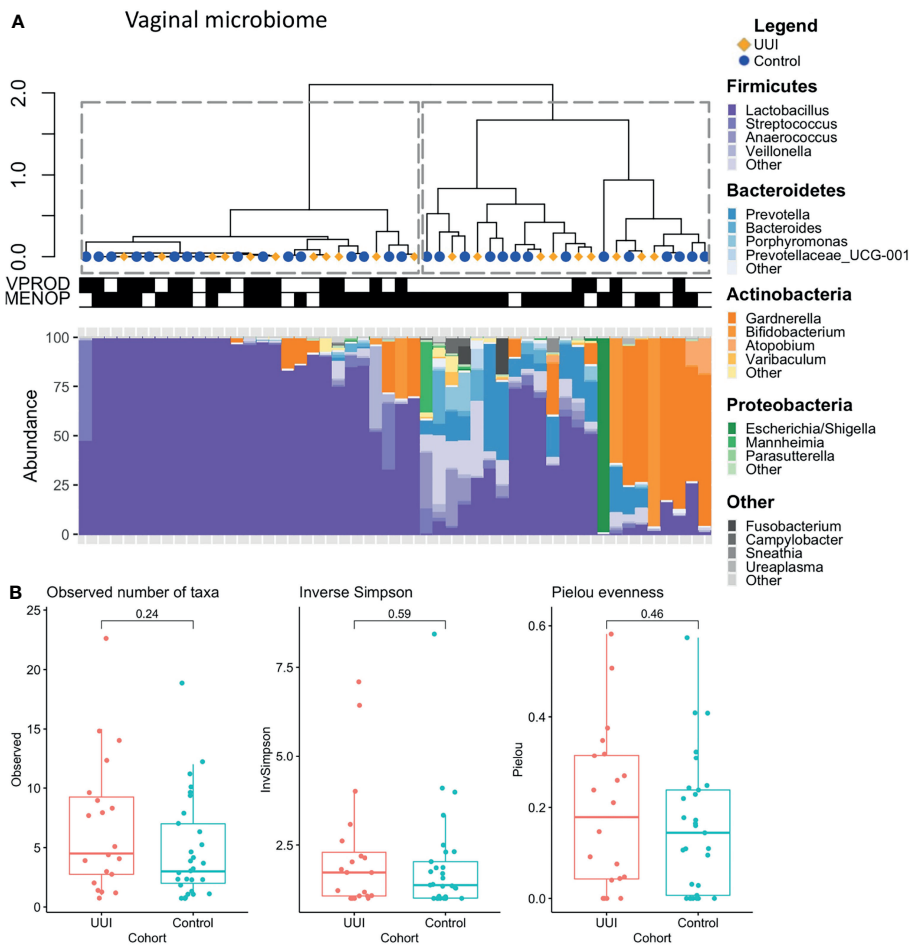

FIGURE 1 | **(A)** Clustering of vaginal microbiome is associated with use of vaginal products. Hierarchical clustering (top) was performed using the Ward's minimum variance method on weighted UniFrac distances between samples. Stacked bar plots (bottom) show relative abundance of vaginal microbiome of women with and without UUI. Dotted lines outline clusters that were chosen based on the Silhouette metric. Black bars underneath annotate for clinical features being TRUE. Vaginal product use was significantly associated with vaginal microbiome cluster ($p = 0.004$), menopausal status was not ($p = 1.0$, Fisher's exact test). Cases are labeled with yellow diamonds and controls are labeled as blue squares. VPROD, any vaginal product use; MENOP, post-menopausal. **(B)** Women with and without UUI do not differ in vaginal microbiome diversity. Alpha diversity is visualized using box-and-whisker plots and measured using observed number of taxa (genera, $p = 0.24$), inverse Simpson Index ($p = 0.59$), and Pielou evenness index ($p= 0.46$). A generalized linear model was used to adjust for age, BMI, and menopause-estrogen status.

Escherichia, *Blautia*, *Faecalibacterium*, *Lachnospiraceae*, *Prevotellaceae*, and others, while the other cluster was primarily dominated by *Lactobacillus* or *Gardnerella*. We tested for associations between the microbiome clusters and clinical/demographic characteristics and found no relationship between cohort status (UUI vs control, $p = 0.76$) or vaginal product use ($p = 0.76$). However, menopausal status did have a significant association with the clusters ($p = 0.01$), with the diverse microbiome cluster being more associated with samples from postmenopausal women. We did not find any significant differences in alpha diversity (**Figure 3B**) or beta diversity in the bladder microbiome of women with and without UUI (**Supplemental Figure 2**, PERMANOVA $p = 0.23$, adjusted for age, BMI, menopause status, and estrogen use).

Similar to the vaginal microbiome analysis, we inferred microbiome-wide interaction network for each cohort group (UUI and control) independently (**Figure 4** and **Table 6**). The basic structure of the bladder microbiome network showed five clustered subgroups of bacteria for women with UUI and eight clusters for controls. Visually and quantitatively using modularity and connectance, we see that the control bladder microbial network is more clustered into smaller microbial groups. The UUI network had fewer genera in the connected network (93 genera in UUI versus 135 genera in controls) and fewer unique bacterial co-occurrences (624 associations in UUI versus 763 associations in controls).

We explored key urinary bacteria in the UUI and control bladder microbial networks using the betweenness centrality measure. Unlike the vaginal microbiome, we found that *Lactobacillus* genera were the central bacteria with the highest centrality measure in both UUI and controls (**Table 7**). Because *Lactobacillus* are central to both UUI and control networks, we

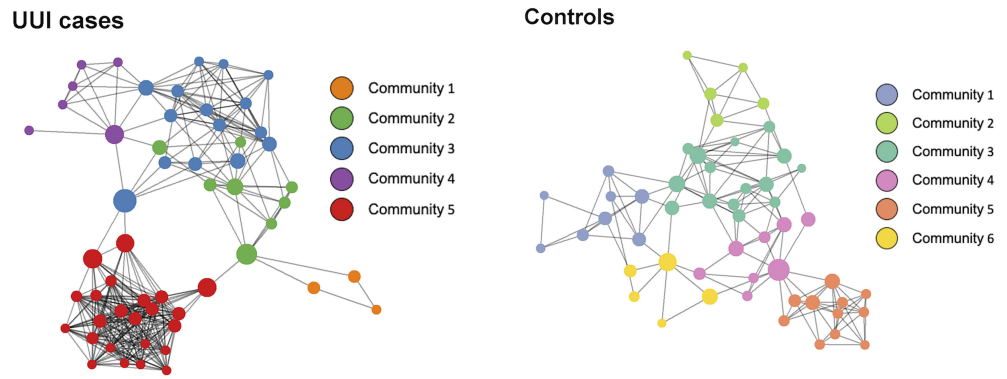

FIGURE 2 | Network visualization of vaginal microbiomes. Each microbiome co-occurrence network, one for the UUI group (left) and one for the control group (right) consists of several bacterial genera (circles, colored by communities of bacteria identified using the InfoMap algorithm and sized by betweenness centrality value) that are connected to other bacterial genera by co-occurrence (edges/lines connecting circles, identified by SparCC correlation).

TABLE 3 | Summary of metrics for vaginal microbiome networks.

	Interpretation	UUI	Control
Number of nodes	Space of co-occurring bacteria to consider	55	52
Number of edges	Number of co-occurrence relationships	343	152
Modularity	Measure of community detection	0.45	0.57
Normalized connectance	Complexity of system	0.23	0.11

TABLE 4 | Betweenness centrality measured of key bacteria in the vaginal microbiome network (defined by bacteria with a betweenness score >0.10).

Genus	UUI	Control
Bacteroides	0.14	0.39
Aerococcus	0.33	0.07
Streptococcus	0.22	0.07
Lactobacillus	0.00	0.19
Mannheimia	0.16	0.04
Prevotellaceae_UCG-001	0.16	0.01
Gardnerella	0.15	0.06
Anaerococcus	0.02	0.15
Fusobacterium	0.00	0.14
Staphylococcus	0.06	0.12

TABLE 5 | Top bacteria correlated with *Aerococcus* in the UUI vaginal network.

Genus	Correlation
Actinomyces	0.46
Staphylococcus	0.41
Helcococcus	0.39
Streptobacillus	0.39
Prevotellaceae	0.36
Gardnerella	0.36
Bacteroides	0.24

explored the microbial associations between the *Lactobacillus* and other genera in each cohort. We found 22 unique genera in UUI and 20 unique genera in the controls that associated with *Lactobacillus* in their respective networks. Both networks shared a total of six common genera that associated with *Lactobacillus–Campylobacter, Dialister*, *Gardnerella*, *Prevotellaceae_NK3B31_group*, *Ureaplasma*, and *Varibaculum*. Among the unique associated genera in UUI, we found that at least a quarter of them give rise to species known to be uropathogens associated with urinary tract infections. These include *Aerococcus*, *Corynebacterium*, and *Escherichia/Shigella* (**Table 8**).

Vaginal and Bladder Microbiomes: Is There an Overlap?

Overall, more bacterial genera were identified from the bladder (131) compared to the vagina (66). The number of shared bacterial genera between the bladder and the vagina differed between women with and without UUI. Women with UUI have a larger number of shared bacterial genera (43%) between the two adjacent ecosystems compared to controls (29%) (**Figure 5** and **Table 9**). Among women with UUI, the top most abundant vaginal bacterial genera that were also present in the bladder include *Lactobacillus*, *Bifidobacterium*, *Gardnerella*, *Prevotella*, *Sneathia*, *Faecalibacterium*, *Varibaculum*, *Actinotignum*, and *Aerococcus*. Of these, only *Gardnerella* was more abundant in the bladder compared to the vagina. Other overlapping genera with higher median abundance in the bladder include *Bacteroides*, *Prevotellaceae_UCG-001*, and *Escherichia/Shigella*. Among controls, the most abundant vaginal bacterial genera that were also present in the bladder include *Lactobacillus*, *Gardnerella*, *Bifidobacterium*, *Atopobium*, *Prevotella*, *Bacteroides*, and *Streptococcus*. Of these, only *Bacteroides* was more abundant in the bladder compared to the vagina. Other top bladder genera with

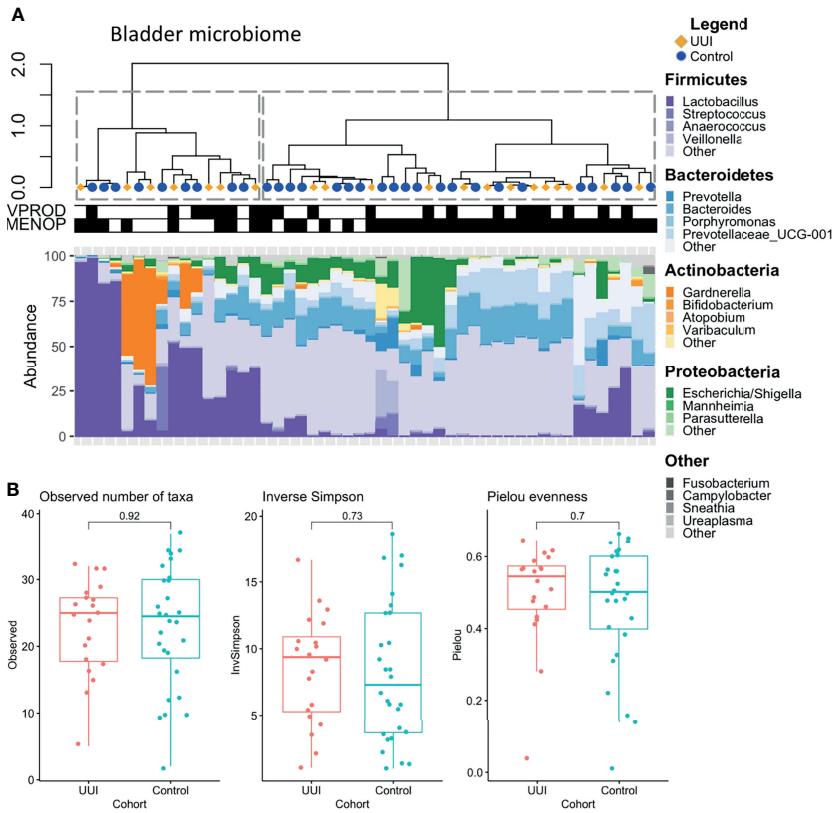

FIGURE 3 | **(A)** Clustering of bladder microbiome is associated with menopausal status. Hierarchical clustering was performed using the Ward's minimum variance method on weighted UniFrac distances between samples. Dotted lines outline clusters that were chosen based on the Silhouette metric. Black bars underneath annotate for clinical features being true. Menopausal status was associated with bladder microbiome cluster (p = 0.01), but vaginal product use was not (p =1.0, Fisher's exact test). Cases are labeled with yellow diamonds and controls are labeled as blue squares. MENOP, post-menopausal. **(B)** Women with and without UUI do not differ in bladder microbiome diversity. Alpha diversity is visualized using box-and-whisker plots and measured using observed number of taxa (genera, p = 0.92), inverse Simpson Index (p = 0.73), and Pielou evenness index (p= 0.70). A generalized linear model was used to adjust for age, BMI, and menopause-estrogen status.

higher median abundance in the bladder compared to vagina include *Prevotellaceae_UCG-001*, *Actinomyces*, and *Faecalibacterium*. Irrespective of overall abundance in the vagina or bladder, the bacteria that has the highest overlap in median abundance in both ecosystems in the UUI group was *Gardrenella* (Vagina: 26.8, Bladder: 55.8) followed by *Lactobacillus* (Vagina: 69.6, Bladder: 6.3) where as in the control group, the bacteria with the highest overlap in median abundance were *Lactobacillus* (Vagina: 74.2, Bladder: 15.5) followed by *Gardrenella* (Vagina: 43.0, Bladder: 3.1).

DISCUSSION

It is generally accepted that the vagina acts as an intermediary between the gut and the bladder and may play a role in the pathogenesis of bladder conditions such as UTI (Czaja et al., 2009). It is also known that disruption of a normal microbiome environment in the vagina is associated with colonization by pathogenic organisms leading to disorders like bacterial vaginosis (Srinivasan et al., 2010), sexually transmitted infections (STIs) (van de Wijgert, 2017; Eastment and McClelland, 2018; Ziklo et al., 2018), and genital herpes infection (Shannon et al., 2017). It is however unclear if and how dysbiosis in the vagina plays a role in more chronic bladder conditions such as UUI.

In this study, we provide a detailed characterization of the differences in both bladder and vaginal microbiomes in a cohort of well-characterized women with and without UUI. More importantly, we go beyond traditional approaches to microbiome analysis by leveraging network-based analysis to look at underlying microbial community dynamics or interactions in these adjacent ecosystems. Network analysis provides a mathematical tool to understand complex systems such as identifying central bacteria that contribute to stability and resilience of ecosystems and their interactions with others in the network (Pielou et al., 2007).

In our study, women with UUI were similar to women without UUI in all clinical and demographic variables except for history of recurrent UTIs. Women with UUI were more likely to have a history of recurrent UTI which is not surprising given the known overlap in symptoms between UTI and UUI and the frequent misdiagnosis of UTI as a result (Nik-Ahd et al., 2018). There is also some evidence that overactive bladder may be

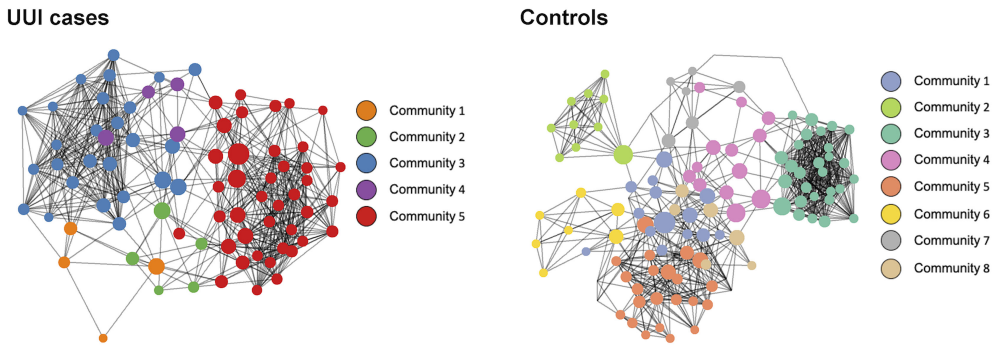

FIGURE 4 | Network visualization of bladder microbiomes. Each microbiome co-occurrence network, one for the UUI group (left) and one for the control group (right) consists of several bacterial genera (circles, colored by communities of bacteria identified using the InfoMap algorithm and sized by betweenness centrality value) that are connected to other bacterial genera by co-occurrence (edges/lines connecting circles, identified by SparCC correlation).

TABLE 6 | Summary of network metrics for bladder microbiome networks.

	Interpretation	UUI	Control
Number of nodes	Space of co-occurring bacteria to consider	93	135
Number of edges	Number of co-occurrence relationships	624	763
Modularity	Measure of community detection	0.46	0.60
Normalized connectance	Complexity of system	0.15	0.09

TABLE 7 | Betweenness centrality measures of key bacteria in the bladder microbiome network (defined by bacteria with a betweenness score >0.10).

Genus	UUI	Control
Lactobacillus	0.14	0.20
Corynebacterium	0.01	0.15
Bifidobacterium	0.02	0.13
Akkermansia	0.00	0.13

TABLE 8 | Top bacteria correlated with *Lactobacillus* in the urinary network.

Genera associated in both groups	
Genus	Correlation
Campylobacter	0.43
Dialister	0.37
Gardnerella	0.28
Prevotellaceae_NK3B31_group	0.34
Ureaplasma	0.34
Varibaculum	0.31
Genera unique to UUI group	
Genus	Correlation
Actinotignum	0.42
Aerococcus	0.32
ASF356	0.39
Corynebacterium	0.33
Erysipelotrichaceae_UCG-003	0.37
Escherichia/Shigella	0.31
Flavonifractor	0.33
Intestinimonas	0.30
Jonquetella	0.42
Lachnospiraceae_NK4B4_group	0.35
Luteibacter	0.32
Marvinbryantia	0.31
Meiothermus	0.40
Oscillibacter	0.41
Paludibacter	0.30
Prevotella	0.30
Ruminococcaceae_NK4A214_group	0.31
Ruminococcaceae_UCG-009	0.24
Ruminococcaceae_UCG-013	0.32
Sneathia	0.30
Tumebacillus	0.38
Tyzzerella	0.24

associated with chronic low-grade colonization by bacteria that are commonly missed on routine cultures (Balachandran et al., 2016). These observations are the impetus for investigators, us included, to look at the role of bladder microbiomes in health and disease (Karstens et al., 2016; Brubaker and Wolfe, 2017; Drake et al., 2017; Aragón et al., 2018).

In our study, we did not see a significant difference in alpha or beta diversity in bladder or vaginal microbiomes of women with and without UUI. This is consistent with our own prior reporting (Karstens et al., 2016) but differed from others who reported increased diversity in UUI (Pearce et al., 2015; Thomas-White et al., 2016). Using silhouette analysis, we showed that the bladder and vaginal microbiomes formed two clusters of bacteria. In the vagina, one cluster was dominated by *Lactobacillus* and the other with mixed bacteria. Presence or absence of UUI did not impact clustering. The only clinical factor that was associated with this clustering was use of "any vaginal product" the week before collection of the vaginal specimen, with more vaginal product use noted in the *Lactobacillus*-dominated group. This observation is important to note and highlights the

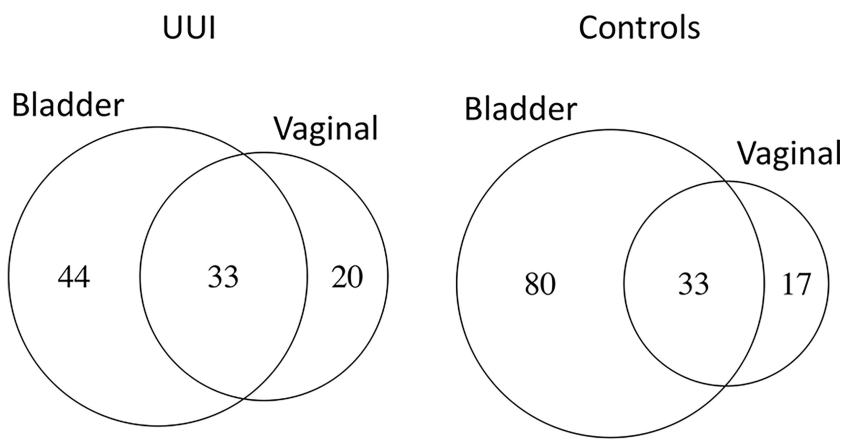

FIGURE 5 | The number of shared bacterial genera between vaginal and bladder microbiomes of women with UUI differ from women without UUI. Women without UUI (controls) have more unique bacteria in the bladder microbiome.

importance of environmental factors such as vaginal product use (douching, lubrication, estrogen cream etc.) that influence vaginal microbiome. This is consistent with the literature highlighting the dynamic nature of vaginal microbiomes and how these ecosystems can be perturbed by factors like menstruation, vaginal products, vaginal infections, hormonal status such as pregnancy, menopause, etc. (Srinivasan et al., 2010; Gajer et al., 2012; DiGiulio et al., 2015). Interestingly, the clustering in the vaginal microbiome was not associated with estrogen use *per se*. The small size of our study (with an even smaller number of women on estrogen therapy) does not allow us to make any definitive conclusion about the association of vaginal or systemic estrogen use and changes in vaginal or bladder microbiomes. This certainly needs further investigation given the promising evidence that vaginal estrogen improves OAB symptoms in post-menopausal women (Cardozo et al., 2004), reduces symptoms associated with genitourinary syndrome of menopause including the risk of UTI (Rahn et al., 2014), and that vaginal estrogen use may increase *Lactobacillus* in the urine of post-menopausal women (Thomas-White et al., 2020). This suggests that modulation of microbiomes is one likely mechanism of action for vaginal estrogens.

Similar to the vagina, silhouette analysis in the bladder microbiome identified two clusters. One cluster contains bladder microbiomes that have a higher proportion of *Lactobacillus* and *Gardnerella*, while the second cluster contains microbiomes with a mixed population of bacteria. This clustering in the bladder microbiome was associated with menopausal status in which post-menopausal women were more likely to have microbiomes without a dominant genus while premenopausal women tended to have microbiomes that were dominated by *Lactobacillus* or *Gardnerella*. This is consistent with prior report that shows higher *Lactobacillus* in the urine of premenopausal women compared to post-menopausal women (Curtiss et al., 2018). In our study, these dominant clusters in the bladder were not associated with presence or absence of UUI. This is in agreement with some prior work using similar-sized cohorts (Pearce et al., 2014), but different from other studies that found several clusters within UUI samples that were dominated by single bacteria such as *Lactobacillus* and *Gardnerella* (Pearce et al., 2015) and studies that identified bacterial community types that were different between women with and without mixed urinary incontinence (Komesu et al., 2018).

One of the unique aspects of this study was the use of network analysis to understand microbial interactions in the bladder and in the vagina. We identified two major microbiome interaction networks in both the vaginal and bladder samples. In the vaginal samples, we identified similar numbers of genera in each network for women with and without UUI. They also have similar numbers of clusters of subgroups in the microbiome-wide networks. However, women with UUI tend to have more unique bacterial associations or co-occurrences compared to controls. In the bladder on the other hand, cases have less than half as many network clusters compared to controls, suggesting that loss of smaller and more specialized microbiome networks may be a characteristic of UUI.

As part of our network analysis, we looked further to identify central bacteria that are defined by the highest betweenness score (centrality measure) and potentially play important roles in stabilizing the whole community. In network analysis, a node (which in our study represents a genus) that has maximum centrality can be thought of as a keystone taxon which maintains the network structure and potentially the function of the ecosystem. This is irrespective of whether or not it is the most abundant bacteria. In our study, we chose the threshold of 0.10 for our betweenness score because 90% of the values for both microbiome networks were lower than this threshold. In the vaginal microbiome network analysis, we found that *Bacteroides* and *Lactobacillus* were the two top central genera in control samples while *Aerococcus* was the central bacteria in women with UUI. *Bacteroides* as a central bacterium in women with normal bladder control is consistent with what we know about the role of

TABLE 9 | Bacteria shared across vaginal and bladder microbial communities in women with UUI and Controls, ranked by median abundance in the vaginal community.

UUI			Controls		
Bacteria	**Vaginal median**	**Bladder median**		**Vaginal median**	**Bladder median**
Firmicutes			**Firmicutes**		
Lactobacillus	69.56	6.27	*Lactobacillus*	74.24	15.46
Faecalibacterium	5.58	5.16	*Streptococcus*	4.04	1.49
Aerococcus	3.7	4.6	*Veillonella*	3.08	1.21
Agathobacter	2.49	3.56	*Peptostreptococcus*	2.18	0.88
Lachnospira	2.49	1.69	*Agathobacter*	1.5	2.91
Ruminiclostridium	2.22	6.62	*Faecalibacterium*	1.45	5.04
Subdoligranulum	2.2	3.06	*Roseburia*	1.28	1.84
Ruminococcus	2.14	2.48	*Enterococcus*	1.21	2.14
Anaerostipes	2.01	1.66	*Ruminococcus*	1.15	2.67
Dialister	1.77	1.88	*Lachnospiraceae NK4A136 group*	1.03	2.31
Lachnospiraceae NK4A136 group	1.31	2.57	*Ruminiclostridium*	1.02	3.48
Fusicatenibacter	1.23	3.18	*Blautia*	0.91	3.86
Roseburia	1.2	3.39	*Dialister*	0.85	1.33
Streptococcus	1.1	0.84	*Lachnospira*	0.85	1.81
Phascolarctobacterium	1.01	1.19	*Ruminococcaceae UCG-002*	0.81	1.56
Blautia	1	3.89	*Staphylococcus*	0.7	0.88
Butyrivibrio	0.9	1.04	*Anaerostipes*	0.61	1.58
Lachnospiraceae ND3007 group	0.83	0.97			
Dorea	0.75	1.16			
Ruminococcaceae UCG-013	0.66	1.26			
Actinobacteria			**Actinobacteria**		
Bifidobacterium	64.35	0.86	*Gardnerella*	43.04	3.09
Gardnerella	26.82	55.84	*Bifidobacterium*	31.7	0.99
Varibaculum	4.98	1.29	*Atopobium*	13.1	1.33
Actinotignum	4.11	0.8	*Actinomyces*	1.49	5.2
			Corynebacterium	0.89	0.71
			Varibaculum	0.86	0.57
Bacteroidetes			**Bacteroidetes**		
Prevotella	16.19	1.57	*Prevotella*	10.09	2.48
Prevotellaceae Ga6A1 group	2.21	2.83	*Bacteroides*	5.32	9.1
Prevotellaceae UCG-001	1.66	13.28	*Prevotellaceae NK3B31 group*	1.77	1.04
Alistipes	0.78	1.57	*Prevotellaceae UCG-001*	1.31	8.52
Bacteroides	0.71	16.39	*Alistipes*	0.81	2.24
Proteobacteria			**Proteobacteria**		
Escherichia/Shigella	1.8	4.63	*Escherichia/Shigella*	1.51	5.08
			Salmonella	1.11	2.32
Tenericutes			**Tenericutes**		
Ureaplasma	1.15	1	*Ureaplasma*	1.6	1.06
Epsilonbacteraeota			**Epsilonbacteraeota**		
Campylobacter	1.66	2.08	*Campylobacter*	1.4	1.87
Fusobacteria			**Verrucomicrobia**		
Sneathia	8.15	0.86	*Akkermansia*	2	2.9

Bacteroides in maintaining health in the urogenital tract. For example, delay in appearance or absence of *Bacteroides* has been observed in the guts of infants born by cesarean section compared to those delivered by the vaginal route, and this has been proposed as one of the etiologies for the higher predisposition of children born by cesarean section to disorders related to poor immune system maturation (Grönlund et al., 1999; Huurre et al., 2008). This seems to indicate that *Bacteroides* in the maternal birth canal (vagina) may have a key role for developing a healthy immune system that may persist in adults. *Lactobacillus*, the second most central vaginal bacteria in our control group, have been known to be a characteristic of a healthy vagina (Ravel et al., 2011; Drell et al., 2013). Our finding shows that beyond being the most abundant bacteria in the healthy vagina, *Lactobacillus* are also the central bacteria in the microbiome-wide network and thus may play a key role in the stability and function of this ecosystem. In our study, we did not perform a species-level analysis and thus are unable to explain the relevance of our findings on known vaginal community state types dominated by various species of *Lactobacillus*, such as *L. crispatus*, *L. gasseri*, *L. iners*, *bacterial vaginosis-associated bacteria* and *L. jensenii*.

In women with UUI, we found that the most central bacteria in the vaginal microbiome network is *Aerococcus*. Some species within this genus, such as *A. urinae*, are increasingly being recognized for their pathologic role in urinary tract disorders such as UTI, OAB, and UUI (Pearce et al., 2014; Kline and Lewis, 2016; Hilt et al., 2020). Among the top genera most associated with *Aerococcus* were *Actinomyces*, *Gardnerella*, *Prevotella*, and *Bacteroides*. *Actinomyces* species have been reported as

associated with genitourinary actinomycosis (particularly in the setting of IUD use) and urinary tract actinomycosis (Huang and Al-Essawi, 2013; García-García et al., 2017). *Gardnerella* and *Prevotella* are well-known pathogens involved in bacterial vaginosis (Randis and Ratner, 2019). These findings seem to suggest that dysbiosis in the vagina characterized by changes in microbiome-wide community structure may be associated with urinary disorders like UUI.

In the bladder microbiome network analysis, *Lactobacillus* was the central genera in both UUI and control groups. Both groups shared *Lactobacillus* association with six common genera, but they each had several unique genera that did not overlap. Interestingly, many of the unique associations seen in women with UUI were with known uropathogens associated with UTI. It is important to note that in the bladder microbiome network analysis, what differentiates women with and without UUI is not the type of central bacteria (*Lactobacillus* for both*)* but rather its association with other bacteria in the network. This emphasizes the role of community network structures in health and disease and the need to go beyond quantification of relative abundance or diversity measures of microbiomes to understand dynamic ecosystems.

Looking at the similarities across the bladder and vaginal microbiomes, our study shows that women with UUI have a larger number of shared bacteria (43%) compared to women without UUI (29%). Interestingly, we found that although the two top bacteria that were most abundantly shared between these two ecosystems were the same between UUI and control samples (*Lactobacillus* and *Gardnerella), Gardnerella* was the most shared in the UUI subjects (24% overlap vs. 8.3%) while *Lactobacillus* dominated in the controls (17.3% overlap vs. 6.7%). Komesu et al. showed an overlap of 60% of the bacterial taxonomic units (genera) between the vagina and the bladder with the most abundant being the genus *Lactobacillus* (2020). Their study used younger participants (average age 53 compared to 61 in our study) and did not distinguish between disease and non-disease states. Others who performed species-level analysis comparing bladder genome samples with publicly available vaginal strains showed that there was an overlap of 23 species between the vagina and the bladder (Thomas-White et al., 2018). However, the majority of the samples in their study were from unrelated individuals in a less well-defined clinical population. Our observation that there is higher overlap in bacterial genera between bladder and vagina in women with UUI compared to controls and that the dominant bacteria involved in this overlap differs between these two groups indicates the possibility for more seeding of potentially pathogenic bacteria from the vagina into the bladder in women with UUI compared to controls. How this contributes to dysbiosis of the entire ecosystem is unclear.

The main limitation of our study is the small sample size that made it difficult to identify all relevant clinical and demographic factors that could influence microbiome community structure. For example, use of various vaginal products could potentially influence vaginal microbiome in different ways. Although there were no women who reported symptoms of vaginal infection in our study, we did not do objective screening for the presence or absence of infection in asymptomatic participants. We also used amplicon sequencing of the V4 region of the 16S rRNA gene to investigate the bladder and vaginal microbiomes. While this method is widely used for microbiome studies, it has known biases and limitations (Caporaso et al., 2012; Karstens et al., 2018; Knight et al., 2018). With this approach, we were limited to genus-level information, and could not robustly assess species-level differences or interactions, which may be of importance for understanding the associations to bladder health of the urogenital microbiome. One strength of our study is the use of a clinically well-characterized and demographically well-matched population of UUI and control subjects and the use of catheter-collected samples. Another, perhaps more important strength of our study is the application of network analysis methods to better understand the microbial community structures and how they differ in the urogenital tract of women with and without UUI. To our knowledge, this is the first time this analysis method has been applied to understand microbiome-wide networks in the urogenital tract of women with and without UUI. This approach provides a powerful tool to understanding the role of microbial communities as a whole in bladder health and disease and how ecosystems may be perturbed by environmental factors.

Conclusion

Our finding highlights the importance of using network analysis techniques to understand microbial community dynamics or interactions. The study also provides further evidence of overlap in microbiomes between proximal ecosystems like the vagina and bladder and how this may affect their role in health and disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://www.ncbi.nlm.nih.gov/sra, PRJNA793927. Code for the analyses presented in this manuscriipt can be found at: https://github.com/KarstensLab/urogenital_microbiome_networks_in_uui.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Oregon Health & Science University, Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RN Conceiving and designing experiment, writing manuscript. EL Data analysis, writing manuscript, editing manuscript. WG Assisting in study design, editing manuscript. LK data Analysis, conceiving and designing experiment, assisting in writing manuscript, editing. MA (Deceased): Assisted in designing and

performing experiment. SD Performing experiment. ED Analyzing Data. All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.759156/full#supplementary-material

Supplementary Figure 1 | Principal Coordinate Analysis (PCoA) for the vaginal microbiome. **(A)** PCoA using Bray-Curtis distance; **(B)** PCoA using unweighted UniFrac distance; **(C)** PCoA using weighted UniFrac distance.

Supplementary Figure 2 | Principal Coordinate Analysis (PCoA) for the urinary microbiome. **(A)** PCoA using Bray-Curtis distance; **(B)** PCoA using unweighted UniFrac distance; **(C)** PCoA using weighted UniFrac distance.

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Choice of 16S Ribosomal RNA Primers Impacts Male Urinary Microbiota Profiling

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Accessibility to next-generation sequencing (NGS) technologies has enabled the profiling of microbial communities living in distinct habitats. 16S ribosomal RNA (rRNA) gene sequencing is widely used for microbiota profiling with NGS technologies. Since most used NGS platforms generate short reads, sequencing the full-length 16S rRNA gene is impractical. Therefore, choosing which 16S rRNA hypervariable region to sequence is critical in microbiota profiling studies. All nine 16S rRNA hypervariable regions are taxonomically informative, but due to variability in profiling performance for specific clades, choosing the ideal 16S rRNA hypervariable region will depend on the bacterial composition of the habitat under study. Recently, NGS allowed the identification of microbes in the urinary tract, and urinary microbiota has become an active research area. However, there is no current study evaluating the performance of different 16S rRNA hypervariable regions for male urinary microbiota profiling. We collected urine samples from male volunteers and profiled their urinary microbiota by sequencing a panel of six amplicons encompassing all nine 16S rRNA hypervariable regions. Systematic comparisons of their performance indicate V1V2 hypervariable regions better assess the taxa commonly present in male urine samples, suggesting V1V2 amplicon sequencing is more suitable for male urinary microbiota profiling. We believe our results will be helpful to guide this crucial methodological choice in future male urinary microbiota studies.

Keywords: urobiome, urinary microbiota, bladder microbiota, 16S amplicon sequencing, 16S rRNA primers

INTRODUCTION

Urine is not sterile (Wolfe and Brubaker, 2015). Modified culture protocols, such as enhanced quantitative urine culture (EQUC), and modern sequencing techniques have now enabled the detection of microbes washed out from the whole urogenital tract (Perez-Carrasco et al., 2021). Because EQUC is labor-intensive and time-consuming (Barnes et al., 2021), culture-independent sequencing-based methods are the main tool to identify microbes inhabiting the urogenital tract.

Microbial communities colonizing the urinary tract (collectively referred to as the urobiome) are influenced by sex, age, environmental factors and even host genetics (Adebayo et al., 2020; Brubaker et al., 2021a; Brubaker et al., 2021b; Perez-Carrasco et al., 2021). Most importantly, recent studies have shown that urobiome dysbiosis is linked to several urological conditions (Brubaker et al., 2021b; Perez-Carrasco et al., 2021), ranging from urinary incontinence (Pearce et al., 2014) to bladder cancer (Wu et al., 2018). Therefore, a comprehensive and systematic characterization of the urobiome in health and disease is fundamental, and may lead to new prevention, diagnosis and treatment strategies for urological pathologies.

Bacteria are the central component of the urobiome and a major technical challenge in DNA-based microbiota studies is the low bacterial biomass of urine samples (Brubaker et al., 2021b). Bladder urine often contains $<10^5$ colony forming units per milliliter, a number at least a million times lower than that found in feces per gram (Karstens et al., 2018). As a consequence, while gut microbiota DNA-based studies are shifting from 16S ribosomal RNA (rRNA) amplicon sequencing towards shotgun metagenomic sequencing - which is problematic with low amounts of input bacterial DNA (Pereira-Marques et al., 2019) -, urinary microbiota profiling still relies on 16S rRNA amplicon sequencing (Cumpanas et al., 2020; Hoffman et al., 2021).

A critical step in 16S rRNA amplicon sequencing studies is the selection of which 16S rRNA hypervariable regions to sequence. 16S rRNA contains nine hypervariable regions (V1-V9) used to determine taxonomic identity and estimate evolutionary relationships between bacteria. Although all nine hypervariable regions are taxonomically informative, the amount and quality of information retrieved varies per region according to the studied environment. For instance, Fadeev et al. (2021) showed that V4V5 is superior to V3V4 for microbiota profiling of environmental arctic samples, and Kameoka et al. (2021) found that V1V2 is more precise than V3V4 for gut microbiota profiling of Japanese individuals. Furthermore, Hoffman et al. (2021) concluded based on a computational analysis that V1V3 and V2V3 allow a more complete assessment of the female urobiome, but validation by sequencing these regions was not performed.

Despite evidence showing that the choice of 16S hypervariable regions in microbiota profiling studies is critical (Cabral et al., 2017; Fadeev et al., 2021; Hoffman et al., 2021; Kameoka et al., 2021; Sirichoat et al., 2021), no study has systematically compared the performance of sequencing different 16S rRNA hypervariable regions for microbial characterization of urine samples. In this work, we compared the performance of different sets of 16S rRNA primers for male urinary microbiota profiling. We collected urine samples from male volunteers by transurethral catheterization and used a 16S rRNA sequencing panel encompassing all nine hypervariable regions. We also combined pairs of non-overlapping 16S rRNA amplicons using bioinformatics reconstruction to evaluate their performance. To identify which primer sets and combinations are best suited for male urinary microbiota profiling, we evaluated the effect of using different primer sets and combinations on metrics such as taxonomic resolution, taxonomic richness and ambiguity. Our results suggest V1V2 amplicon sequencing is more suitable for male urinary microbiota studies. We also observed marginal gains in taxonomic richness when using pairs of amplicons, which may not compensate for the higher costs of sequencing multi-amplicon libraries.

MATERIALS AND METHODS

Sample Collection

Twenty-two urine samples were collected from 14 male volunteers between March 2019 and November 2020. Samples were collected by a trained nurse in sterile urine containers during catheterization for BCG instillation in volunteers with non-muscle invasive bladder cancer or for transurethral resection in volunteers with benign prostatic hyperplasia (**Table S1**). Since the benefit of using preservatives is limited for samples stored at colder temperatures (Jung et al., 2019), samples were stored without preservative at -80°C until DNA extraction.

DNA Extraction

Urine samples were thawed at room temperature, and up to 40 ml of urine was used for DNA extraction. Urine samples were centrifuged for 15 min at 10°C and 3000 g, and the supernatant was discarded sparing 10 ml of urine (containing a pellet). This content was transferred to 15 ml tubes, and centrifugation was repeated (15 min; 10°C; 3000 g). Approximately 1 ml of urine (containing the pellet) was resuspended in 3 ml phosphate-buffered saline (PBS) and centrifugation was repeated (15 min; 10°C; 3000 g). The supernatant was discarded leaving 1 ml of sample in the tube. Samples and the DNA extraction negative control (1 ml PBS) were processed for DNA extraction using the QIAamp DNA Microbiome kit (Qiagen, Hilden, Germany) following the manufacturer's protocol (*Depletion of Host DNA* protocol).

Library Preparation and Sequencing

Twenty-four multi-amplicon libraries were prepared using the QIAseq 16S/ITS Screening Panel kit (Qiagen, Hilden, Germany) as outlined in **Figure S1**. These libraries were prepared using 22 urine DNA samples, the DNA extraction negative control and the QIAseq 16S/ITS Smart Control (Qiagen, Hilden, Germany), a synthetic DNA sample used both as positive control for library preparation and sequencing, and as control for the identification of contaminants. DNA concentration was determined using the Qubit dsDNA HS Assay kit and Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Next, the fungal taxonomic marker internal transcribed spacer (ITS) and six 16S rRNA amplicons, spanning all nine hypervariable regions (V1V2, V2V3, V3V4, V4V5, V5V7 and V7V9), were amplified by PCR. The ITS region was poorly amplified since we used a DNA extraction protocol which depletes eukaryotic DNA.

Sequences originated from the ITS amplicon were therefore discarded. PCR primers and their properties [estimated with OligoCalc (Kibbe, 2007)] are provided in **Table S2**. Amplifications were carried out in three independent reactions with primers multiplexed by the manufacturer. For samples in which DNA concentration was ≥0.25 ng/ul, 1 ng of DNA was used as template, and for samples with <0.25 ng/ul, 4 ul of DNA was used. Cycling conditions were: 95°C for 2 min; 20 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 2 min; and 72°C for 7 min. PCR products from the same sample were pooled and purified twice using QIAseq beads (Qiagen, Hilden, Germany). Dual-index barcodes and adapters were added to amplified products through a second-round of PCR using the QIAseq 16S/ITS 96-Index I array (Qiagen, Hilden, Germany). Cycling conditions were: 95°C for 2 min; 19 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 2 min; and 72°C for 7 min. After an additional purification using QIAseq beads, the presence of target sequences was evaluated with the Agilent Bioanalyzer 2100 System using the Agilent DNA 1000 kit (Santa Clara, CA, USA). Finally, we quantified the libraries using the NEBNext® Library Quant Kit for Illumina (New England Biolabs, Ipswich, MA, USA), size-correcting for the average length reported in the Bioanalyzer report considering a 400-700 bp quantification window. Libraries were normalized to 2 nM and sequenced using the MiSeq Reagent Kit v3 (600-cycle) (Illumina, San Diego, CA, USA) following the 2 x 276 bp paired-end read protocol.

Read Processing

Paired-end reads were library demultiplexed and adapters were removed in the Illumina BaseSpace Sequence Hub. Each library was amplicon demultiplexed using cutadapt (v3.4) (Martin, 2011), generating two FASTQ files (with forward or reverse reads) for every library-amplicon combination. FASTQ files from the same amplicon were grouped in QIIME 2 artifacts and processed as independent datasets (hereinafter referred to as amplicon-specific datasets) using QIIME 2 (Bolyen et al., 2019).

Using DADA2 (Callahan et al., 2016) (*q2-dada2* QIIME 2 plugin), reads were filtered based on default quality criteria, denoised and truncated (at the first instance of median quality score <30) to remove low quality bases at 3' ends. Next, paired-end reads were merged using DADA2 to produce amplicon sequence variants (ASVs). Finally, chimeric ASVs were filtered using VSEARCH (Rognes et al., 2016) (*q2-vsearch* QIIME 2 plugin) and the SILVA database (v138) (Quast et al., 2013) as reference.

Taxonomic Assignment, Nomenclature Homogenization and Contaminant Removal

Custom slices of the SILVA database (v138) for each amplicon were generated using RESCRIPt (Robeson et al., 2021) (*q2-rescript* QIIME 2 plugin). Low-quality reference sequences were removed, identical reference sequences were dereplicated and 16S rRNA hypervariable regions were selected using primer sequences from the first-round of PCR as target sequences. Only selected regions within a reasonable length-range (100-600 nt) were kept in the final amplicon-specific databases. To achieve a more accurate taxonomic assignment for each amplicon-specific dataset (Werner et al., 2012), amplicon-specific Naive-Bayes-based taxonomic classifiers trained in amplicon-specific databases were built using the *q2-feature-classifier* QIIME 2 plugin (Bokulich et al., 2018). Finally, taxonomic assignment of ASVs was performed using amplicon-specific databases and classifiers.

Assigned taxonomies often contain incomplete information or generic proxies, especially at species level. To homogenize taxonomic nomenclature and to prevent inflation of taxonomic richness at species level, we replaced missing data, generic proxies (terms including "_sp.", "uncultured", "metagenome", or "human_gut") and ambiguous taxonomic entries (e.g., "phylum: Bacteroidota|Proteobacteria") by the lowest taxonomic level with complete nomenclature and the corresponding taxon [e.g. "(…) genus: Streptococcus; species: uncultured_bacterium" is replaced by "(…) genus: Streptococcus; species: Genus_Streptococcus"].

Next, we filtered non-bacterial and bacterial contaminants using taxonomic and abundance information. Non-bacterial contaminants were filtered by removing ASVs classified as not being from bacterial origin (taxonomy assigned to mitochondria, chloroplast or unassigned kingdom). Bacterial contaminants were identified using the R package *decontam* (Davis et al., 2018). Briefly, using the DNA extraction negative control and QIAseq 16S/ITS Smart Control libraries as controls for contaminants, we tested whether each ASV was a contaminant by combining frequency and prevalence *decontam* methods. Due to the limited number of DNA extraction negative control libraries, there was limited statistical power to identify contaminants exclusively from abundance data. Therefore, we evaluated manually if potentially contaminant ASVs ($P < 0.25$) had been previously described as belonging to human microbiotas by searching the taxon associated with such ASVs at PubMed (search in May 2021). Potentially contaminant ASVs whose taxonomy had not been previously described in urine [namely, *Pelomonas*, which is a known laboratory contaminant (Salter et al., 2014), *Mycoplasma wenyonii* and *Candidatus Obscuribacter* ASVs] were considered true bacterial contaminants and were removed from all amplicon-specific datasets.

ASVs from amplicon-specific datasets after contaminants removal were also assigned (as described for SILVA) using the Greengenes (v13.8) (McDonald et al., 2012) and the NCBI 16S RefSeq (O'Leary et al., 2016) to evaluate the impact of using alternative reference databases in taxonomic resolution.

Sidle-Reconstruction of Amplicons Combinations

The Short MUltiple Reads Framework (SMURF) algorithm (Fuks et al., 2018) as implemented in Sidle (SMURF Implementation Done to acceLerate Efficiency) (Debelius et al., 2021) was used to reconstruct datasets combining all six 16S rRNA amplicons. The *q2-sidle* QIIME 2 plugin was used (as

described below) with amplicon-specific datasets after contaminants removal.

For ASVs in each amplicon-specific dataset to have a consistent length (as demanded by SMURF algorithm), ASVs were truncated at 300 nt. Reference sequences in the amplicon-specific databases generated previously were also truncated at 300 nt. For each truncated amplicon-specific database, regional k-mers were aligned (with 5 nt maximum mismatch) and a reconstructed database incorporating all amplicon-specific databases was built. Next, we reconstructed the abundance (0 minimum number of counts) and the taxonomic table incorporating all amplicon-specific datasets. Finally, we removed all libraries classified as defective and homogenized taxonomic nomenclature as previously described. Sidle-reconstructed datasets combining pairs of amplicons were built through an analogous pipeline.

Microbiota Analyses

Amplicon-specific datasets were normalized prior to diversity analyses by Scaling with Ranked Subsampling (Beule and Karlovsky, 2020) using the R package *SRS* (Heidrich et al., 2021). The number of reads of the library with the lowest number of reads per dataset was used as normalization cutoffs. The normalized amplicon-specific datasets were used to compute taxonomic and ASV richness (where richness is defined as the number of different observed features per dataset), and Faith's phylogenetic diversity index (Faith, 1992) using the R package *picante* (Kembel et al., 2010). Compositional dissimilarity between samples (beta-diversity) was estimated using either Bray-Curtis (Bray and Curtis, 1957) or Jaccard (Jaccard, 1901) indices using the R package *phyloseq* (McMurdie and Holmes, 2013).

ASVs were aligned using the R package *DECIPHER* (Wright, 2016) to calculate the entropy per nucleotide for each dataset, and the entropy score was calculated using the R package *Bios2cor* (Taddese et al., 2021).

Genera intersections between datasets were determined using the R package *UpSetR* (Conway et al., 2017). Taxonomic trees were generated using the R package *metacoder* (Foster et al., 2017) employing the Reingold-Tilford layout. Only the 32 most abundant taxa were shown when plotting taxa relative abundances (based on minimum relative abundance in at least one sample, which is adjusted for each plot).

Ambiguity was estimated using the abundance output tables generated using Sidle. In these tables, the number of potential 16S rRNA source sequences for each feature is provided. For each dataset, ambiguity was calculated as the sum of the log of the number of potential 16S rRNA source sequences for each feature in the abundance table over the total number of features in the abundance table. To calculate the ambiguity for amplicon-specific datasets (not generated by Sidle), Sidle abundance tables for each amplicon were built as described in the previous section.

The full bioinformatics pipeline and R scripts (R Core Team, 2021) used for plotting [mainly with the R package *ggplot2* (Valero-Mora, 2010)] are available at https://github.com/vitorheidrich/urine-16S-analyses.

RESULTS

Sequencing Output and Taxonomic Resolution

We were able to amplify target sequences from 18 out of the 22 (82%) urine samples. In total we generated 20 amplicon libraries spanning all 16S rRNA hypervariable regions for urinary microbiota profiling (18 libraries from urine samples and libraries for the DNA extraction negative control and the QIAseq 16S/ITS Smart Control). A total of 13,638,685 reads were generated from urine sample libraries (median per library: 609,902; range: 383,982-1,489,196), out of which ~59% were short unspecific reads not associated with any of the amplicons of interest (the read length distribution of each amplicon-specific dataset is shown in **Figure S2**). After amplicon demultiplexing, each amplicon-specific dataset was analyzed in parallel. The total number of reads generated for each amplicon-specific dataset varied between 668,509 (V3V4) and 1,674,525 (V4V5) (**Figure 1A**; **Table S3**). After read filtering and removal of contaminants (see Methods), amplicon-specific datasets showed on average a 28% decrease in the number of reads (**Figure 1A**; **Table S3**). The number of reads removed at each step in our bioinformatics pipeline is detailed in **Table S3**.

Despite an overall balanced relative abundance of reads for each amplicon-specific dataset (**Figure 1B**), some libraries presented a disproportionate number of reads for a particular amplicon (**Figure 1C**). Specifically, libraries #2 and #3 showed a high proportion (>⅓) of V1V2 and V2V3 reads, respectively. We also noted that, despite the high median total number of reads generated for each library (204,433), the extremes varied by orders of magnitude (from 5,366 to 504,852 reads), so that the library with the lowest number of reads (#1) had less than 1,000 reads in 4 out of 6 amplicon-specific datasets. These disparities lead us to remove libraries #1, #2 and #3 from further analyses to prevent the introduction of bias due to low-quality libraries. Finally, we confirmed that the remaining libraries achieved satisfactory sequencing depth by calculating the Good's coverage (Good, 1953) (~100% for all samples) and drawing rarefaction curves (**Figure S3**) for each amplicon-specific dataset.

Within these refined datasets, virtually all sequences in V1V2, V2V3 and V3V4 datasets received a taxonomic assignment up to genus level (**Figure 1D**). On the other hand, V4V5 and V5V7 showed a marked decrease in the percentage of assigned sequences at genus level, suggesting a lack of taxonomic resolution for relatively abundant taxa. Taxonomic assignment up to species level was more rarely achieved overall, but V1V2 and V2V3 datasets showed a noticeably higher percentage of sequences assigned up to species level compared to other amplicons (19.7% and 21.8%, respectively). Importantly, we obtained similar results when analyzing taxonomic resolution using NCBI 16S RefSeq or Greengenes as reference databases (**Figure S4**), confirming that these results are not notably influenced by the database used.

In summary, our results indicate that the protocol used herein is suitable for male urinary microbiota characterization, providing enough sequencing depth to assess several amplicons

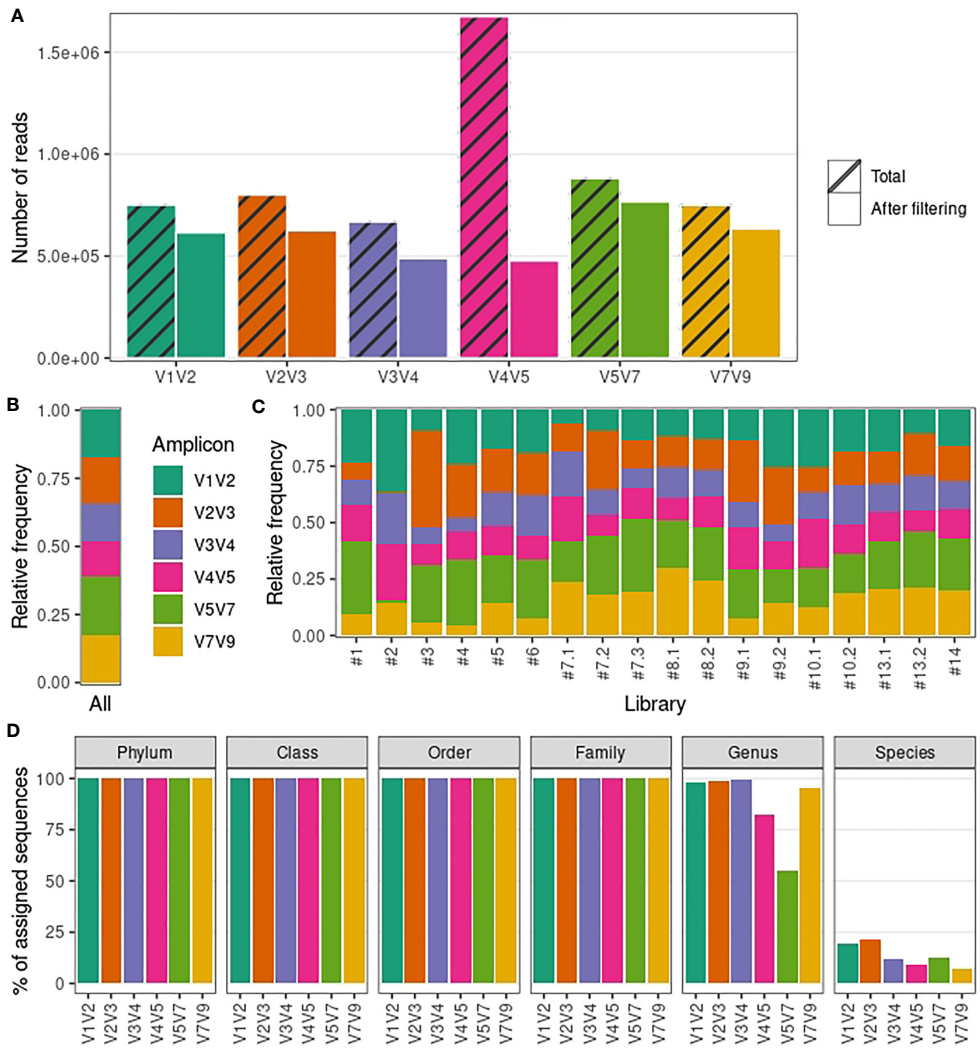

FIGURE 1 | Sequencing output and taxonomic resolution for each 16S rRNA amplicon-specific dataset. **(A)** Number of reads generated and retained after filtering steps for each amplicon-specific dataset. **(B)** Relative frequency of reads retained after filtering steps averaged over all libraries for each amplicon-specific dataset. **(C)** Relative frequency of reads retained after filtering steps per library for each amplicon-specific dataset. **(D)** Percentage of sequences with assigned taxonomy (per taxonomic level) for each amplicon-specific dataset.

simultaneously. We also confirmed that 16S rRNA hypervariable regions sequencing of male urine samples can provide reliable taxonomic information up to genus level. However, taxonomic resolution varies along the 16S rRNA hypervariable regions, with V1V2 and V2V3 achieving the highest taxonomic resolution when considering genus and species levels together.

Richness Across 16S rRNA Amplicon-Specific Datasets

Next, we evaluated ASV and taxonomic (phylum to species level) richness for each amplicon-specific dataset (**Figures 2A, B**). V1V2 and V3V4 datasets showed the highest ASV richness, while V4V5 and V7V9 presented a markedly lower ASV richness (**Figure 2A**). There was no correlation between ASV richness per dataset and the median ASV length per dataset (Spearman ρ = -0.37, P = 0.47). The ASV length distribution of each amplicon-specific dataset is shown in **Figure S5**. At phylum and class level, all amplicons showed a remarkably similar richness (**Figure 2B**), with 6 phyla and 10 classes observed for all datasets, except for the V3V4 dataset (7 phyla and 11 classes). At lower taxonomic levels, differences between amplicons emerged, with the V1V2 dataset showing consistently the highest taxonomic richness from order to species level (**Figure 2B**). There was no correlation between taxonomic richness per dataset and the median ASV length per dataset (**Table S4**).

As expected from its higher ASV richness, V1V2 showed the highest taxonomic richness at genus level. However, we noticed that ASV richness did not always translate into taxonomic richness. For instance, V3V4 goes from the 2nd to the 4th position when richness was assessed at genus level instead of

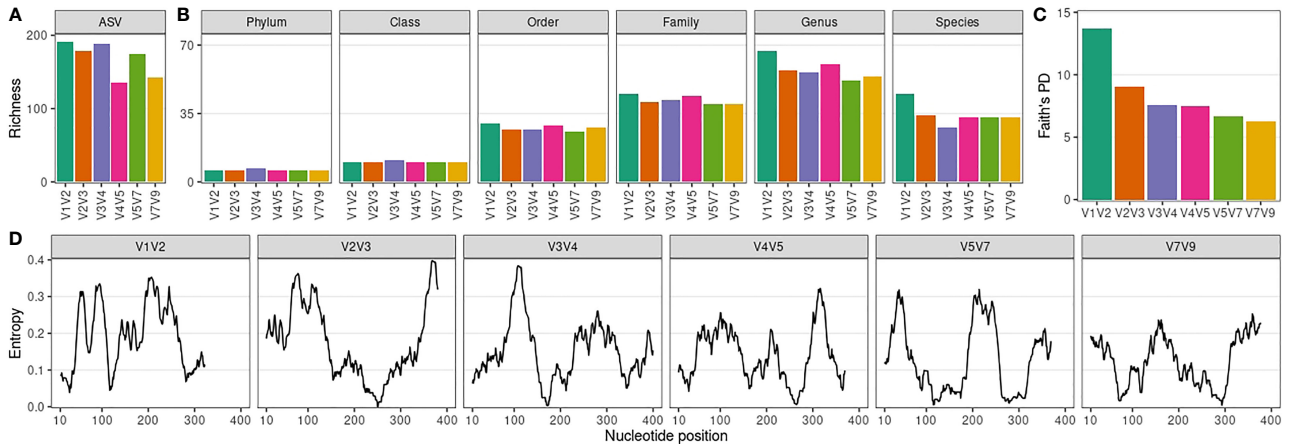

FIGURE 2 | Richness and phylogenetic diversity across 16S rRNA amplicon-specific datasets. **(A)** Amplicon sequence variant (ASV) richness per amplicon-specific dataset. **(B)** Taxonomic richness (phylum to species level) per amplicon-specific dataset. **(C)** Faith's Phylogenetic Diversity (PD) across amplicon-specific datasets. **(D)** Sequence variability (entropy) along ASVs nucleotide positions (20-nucleotides rolling average) for each amplicon-specific dataset. Only nucleotide positions up to the median ASV size per amplicon-specific dataset are considered.

ASV level, suggesting that part of its ASVs correspond to ASVs phylogenetically close to other ASVs observed in the dataset, which do not contribute to increase taxonomic richness. Indeed, V3V4 ASVs showed a much lower phylogenetic diversity compared to V1V2 ASVs (**Figure 2C**). In fact, there is a decreasing trend in phylogenetic diversity along the 16S rRNA hypervariable regions, which is in line with the sequence variability (entropy) observed for each amplicon-specific dataset (**Figure 2D**). There was no correlation between ASV phylogenetic diversity and the median ASV length (Spearman ρ = -0.09, P = 0.92).

Together, our results indicate that V1V2 is the most informative 16S rRNA amplicon in terms of taxonomic richness and phylogenetic diversity for male urinary microbiota characterization.

Taxonomic Composition Across 16S rRNA Amplicon-Specific Datasets

The phyla Actinobacteriota, Bacteroidota, Firmicutes, Fusobacteriota and Proteobacteria were detected in all amplicon-specific datasets. However, some phyla were detected exclusively in a subset of them (**Figure S6A**). Therefore, we analyzed how taxa detection varied across amplicon-specific datasets at genus level. The full picture of the genera detected in each amplicon-specific dataset is provided in **Figure S6B**. Taxonomic trees depicting the contribution of each taxon (tree nodes) to the genera detected in each dataset are provided in **Figure S7**.

When evaluating the intersection of genera present in each amplicon-specific dataset (**Figure 3A**), we see that 27 genera were detected in all amplicon-specific datasets. The next larger subgroup, composed of 15 genera, comprises genera detected exclusively in the V1V2 dataset. Noteworthy, the V1V2 dataset is the only amplicon-specific dataset without exclusively undetected genera, meaning all undetected genera in the V1V2 dataset were also undetected in at least another amplicon-specific dataset. All other datasets also presented "exclusive" genera, which summed up to 31 genera detected in a single amplicon-specific dataset.

Due to such substantial differences, we aimed to assess how the choice of amplicon affects taxonomic profiles. To do so, we used beta-diversity analysis to evaluate whether the taxonomic composition of a given sample is similar to itself irrespectively of the amplicon used for characterization (**Figure 3B**). For all taxonomic levels, using either Bray-Curtis or Jaccard beta-diversity indices, the compositional dissimilarities within samples (same sample profiled with different amplicons) are significantly lower than between samples, suggesting that the choice of amplicons will marginally impact the overall taxonomic compositions, especially at higher taxonomic levels.

The robustness of the taxonomic profile obtained irrespectively of the amplicon of choice can be further contemplated by the similar genera relative abundance profile (averaged over all samples) obtained for each amplicon-specific dataset (**Figure 3C**). In **Figure 3C**, there is an apparently disparate average taxonomic composition for V4V5 and V5V7 datasets. However, this is mainly due to loss of taxonomic resolution for some taxa, with ASVs otherwise classified as genera *Variovorax* and *Klebsiella* being only resolved up to family level (Comamonadaceae and Enterobacteriaceae, respectively) in these datasets. This loss of taxonomic resolution is also observed for *Halomonas* ASVs, which were classified as so in V4V5, V5V7 and V7V9 datasets, but as "Family_Halomonadaceae" in the remaining ones. This phenomenon is even more evident when evaluating taxa relative abundances per sample for each dataset at different taxonomic levels (**Figure S8**), with examples of higher taxonomic resolution at species level (e.g. for *Staphylococcus* sp. in V1V2 and V2V3 datasets).

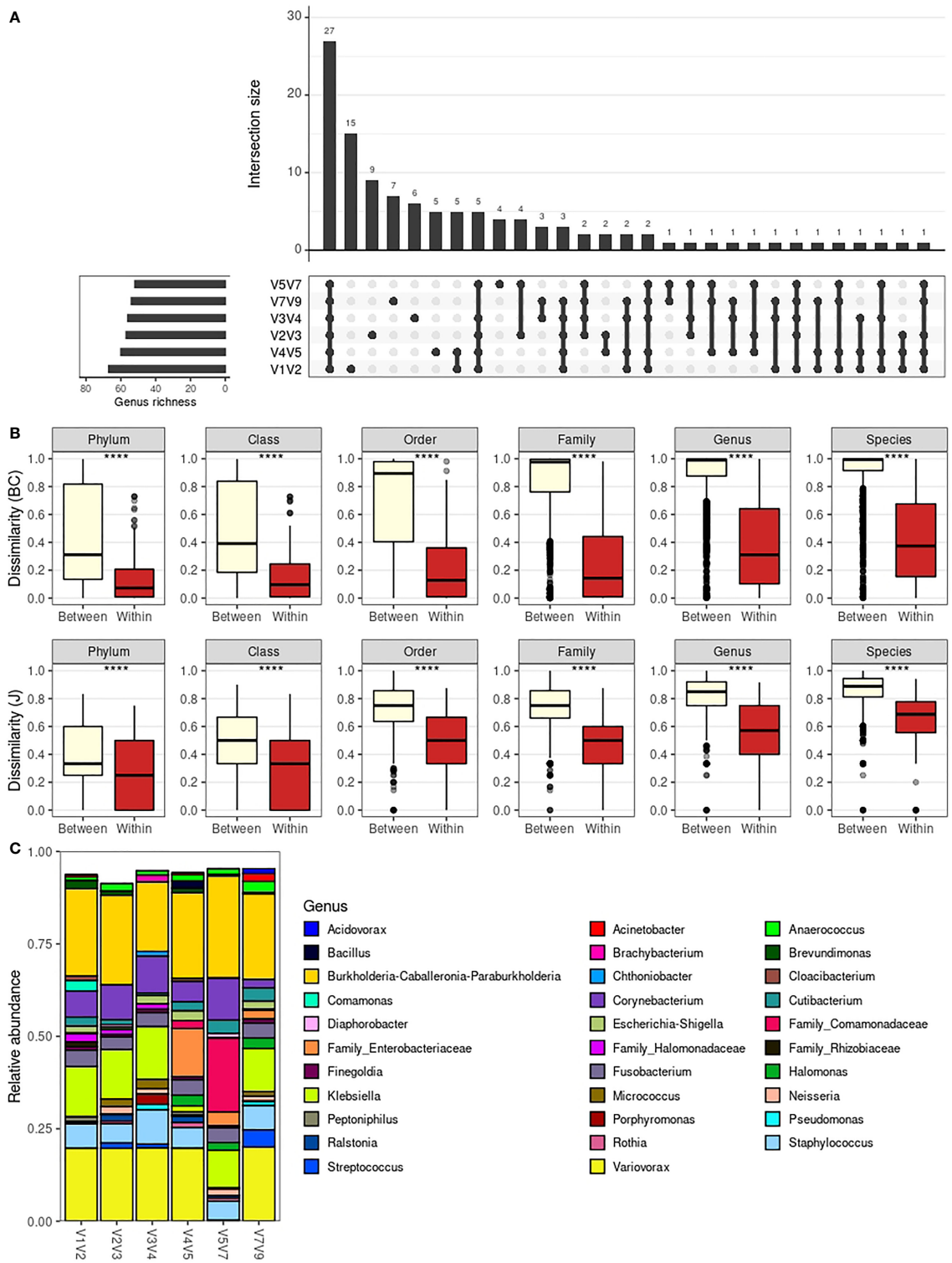

FIGURE 3 | Taxonomic composition across 16S rRNA amplicon-specific datasets. **(A)** Barplot depicting intersections between the genera detected in each amplicon-specific dataset. Total rIchness at genus level is shown in the lower-left subplot. **(B)** Boxplot comparing dissimilarities between different libraries and within the same libraries as profiled with different amplicons. Dissimilarity metrics considered are Bray-Curtis (BC) and Jaccard (J). Statistical significance was evaluated by the Mann-Whitney U test. The boxes highlight the median value and cover the 25th and 75th percentiles, with whiskers extending to the more extreme value within 1.5 times the length of the box. **(C)** Average genera relative abundance per amplicon-specific dataset. Only the 32 most abundant genera are shown (based on minimum relative abundance in at least one sample, which is adjusted for each plot). ****$P < 0.0001$.

We also see from **Figure S8** that the substantial average relative abundance of *Variovorax* and *Klebsiella* in **Figure 3C** is caused by a significant presence of this genera in a small number of low diversity samples (e.g., samples #13.1 and #8.1, respectively) rather than by their presence in a large number of samples. This points to the fact that these averaged profiles, while useful as an analytical tool, do not represent the average male urinary microbiota. Due to our limited sample size, averaged profiles are highly impacted by extreme taxonomic compositions in our cohort.

Despite small variations in taxonomic resolution across amplicon-specific datasets for specific taxa, the overall taxonomic composition of urinary samples is similar independently of the amplicon of choice. Still, each amplicon is able to capture a different subset of the taxa, with V1V2 providing the highest number of exclusively detected genera. These results are in line with the higher genus richness observed for the V1V2 dataset and indicate that V1V2 better captures the actual microbiota composition of male urinary samples.

Comparison With Sidle-Reconstructed Datasets

We next evaluated how the taxonomic richness and composition differ when considering a single amplicon-specific dataset vs. the Sidle-reconstructed taxa abundance table, which incorporates all amplicon-specific datasets. This "full" dataset can then serve as a compiled reference for male urinary microbiota analysis. We also used Sidle to reconstruct taxa abundances for the following pairs of non-overlapping amplicons: V1V2-V4V5, V1V2-V5V7, V1V2-V7V9, V2V3-V5V7, V2V3-V7V9 and V4V5-V7V9.

As expected, there is a considerable gain in richness in the full dataset, mainly at species level, with 3.9x more species observed in the full dataset when compared to amplicon-specific datasets (**Figure 4A**). The use of pairs of amplicons also increases richness on average, but to a lower extent (**Figure S9A**), with V2V3-V7V9 combination providing the greatest increase in richness at species level (1.9x). This result can be explained by a more complete taxonomic assignment being achieved for a greater proportion of sequences in the full dataset (**Figure S9B**). Indeed, better taxonomic resolution observed for the Sidle-reconstructed datasets is due to the lower ambiguity (see Methods) in taxonomic assignment (**Figure 4B**). Noteworthy, the V1V2 dataset shows the lowest ambiguity when comparing only single amplicon-specific datasets.

Once again, the overall taxonomic composition is similar between datasets at genus level (**Figure 4C**). However, we see cases in which identification at species level was only possible in Sidle-reconstructed datasets (e.g., *Klebsiella pneumoniae* was identified in the full dataset and in most of the pairs of non-overlapping amplicons combinations) (**Figure S9C**). The taxa relative abundance per sample for the Sidle-reconstructed datasets at different taxonomic levels is provided in **Figure S10**.

Overall, the combination of amplicons through Sidle increases the taxonomic resolution achievable from 16S rRNA amplicon sequencing. However, the increase of combining pairs of amplicons is modest compared to the full reconstruction using all 16S hypervariable regions, which increases up to 4-fold the number of species detected. Still, this has limited impact in the taxonomic compositions, as evaluated by comparison with the taxonomic profiles generated by single amplicons. Once again, V1V2 stands out as the least ambiguous amplicon for male urinary microbiota characterization.

V1V2 Taxonomic Composition

Due to the great number of taxa identified in the V1V2 dataset, we next investigated whether these taxa are commonly associated with the urogenital microbiota. In our cohort, six phyla were detected using V1V2 amplicon sequencing: Proteobacteria (72.4% of the sequences), Firmicutes (11.3%), Actinobacteriota (9.6%), Fusobacteriota (4.5%), Bacteroidota (2.3%) and Campilobacterota (<0.1%). All of these phyla have been previously reported in studies using catheterized urine samples (Mansour et al., 2020; Hussein et al., 2021; Oresta et al., 2021). Only one of such studies reported the overall phyla abundance. The top-three most abundant phyla in Mansour et al. (2020) were Firmicutes, Proteobacteria and Actinobacteriota. However, their cohort included females, which are known to have a Firmicutes-enriched urogenital microbiota due to the high abundance of lactobacilli (Pearce et al., 2015). In fact, a study with voided urine specimens from male bladder cancer patients found the same top-three most abundant phyla as described in this study (Wu et al., 2018).

Next, we examined the 15 genera detected exclusively in the V1V2 dataset. The average relative abundance of these genera varied between <0.001% (*Alkalibacterium* and *Jeotgalibaca*) and 2.9% (*Comamonas*), summing up to ~4% of the bacterial microbiota exclusively detected by V1V2 16S amplicon sequencing (**Table S5**). Due to the overall low relative abundance of these genera, we excluded the possibility of them being contaminants by searching the literature for the presence of these genera in urine samples. Briefly, 12 out of the 15 (80%) genera exclusively detected in the V1V2 dataset have been previously detected in human samples, and 10 out of 12 (83%) have been associated with urinary infections or detected in urogenital microbiota (**Table S5**). The three genera that were not previously detected in human microbiotas (*Alkalibacterium*, *Chromohalobacter*, *Salipaludibacillus*) sum up to only <0.01% average relative abundance in the V1V2 dataset. They have been described mainly as environmental high salt tolerant bacteria (Ventosa et al., 1989; Yumoto et al., 2014; Sultanpuram and Mothe, 2016), indicating they may represent undetected contamination or taxonomic misclassifications.

Finally, we compared our results with 16S amplicon sequencing-based microbiota studies using catheterized urine samples. In Forster et al. (2020), the urinary microbiota from 34 children with neuropathic bladder was characterized by V4 amplicon sequencing. More than 75% of the samples were dominated (relative abundance >30%) by family Enterobacteriaceae members, but the genera involved in this phenomenon could not be determined due to limited taxonomic resolution. We also observed dominance by Enterobacteriaceae members in this cohort (samples #8.1 and #8.2; **Figure S8**), but because all nine Enterobacteriaceae ASVs in

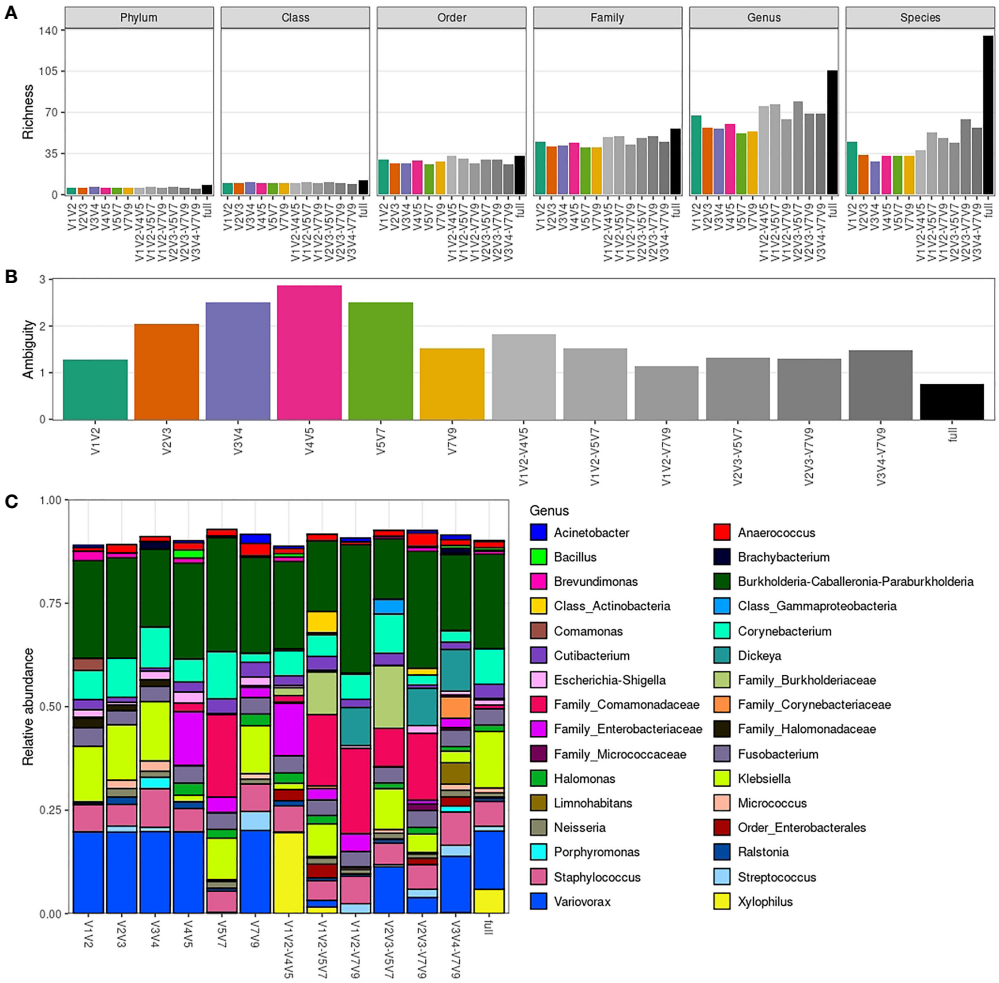

FIGURE 4 | Richness and taxonomic composition of Sidle-reconstructed datasets. **(A)** Taxonomic richness (phylum to species level) per amplicon-specific or Sidle-reconstructed dataset. **(B)** Ambiguity in taxonomic assignment per amplicon-specific or Sidle-reconstructed dataset. **(C)** Average genera relative abundance per amplicon-specific or Sidle-reconstructed dataset. Only the 32 most abundant genera are shown (based on minimum relative abundance in at least one sample, which is adjusted for each plot).

the V1V2 dataset were classified up to genus level (either to *Klebsiella* or *Escherichia-Shigella*), we were able to determine that *Klebsiella* sp. were responsible for this phenomenon. Noteworthy, in V4V5 and V5V7 datasets, family Enterobacteriaceae ASVs could not be classified up to genus level (**Figure S8**), recapitulating the limited taxonomic resolution for family Enterobacteriaceae observed in the aforementioned study.

Together, these data corroborate that V1V2 amplicon sequencing can provide reliable and richer taxonomic information for microbiota profiling of catheterized urine samples from males.

DISCUSSION

Many studies have compared the performance of different sets of 16S rRNA primers for microbiota profiling in different environments (Cabral et al., 2017; Fadeev et al., 2021; Kameoka et al., 2021; Sirichoat et al., 2021). These studies consistently demonstrated that the choice of the 16S rRNA primer set can significantly influence the analysis of microbiota diversity and composition. Apart from a recent study evaluating the female urobiome (Hoffman et al., 2021), similar studies for urinary microbiota profiling are lacking. As reviewed by Cumpanas et al. (2020), out of 38 urobiome studies, 17 evaluated the V4 and 4 evaluated the V3V4 16S rRNA hypervariable regions. This is probably because these amplicons are commonly used in 16S rRNA amplicon sequencing commercial kits. It is also worth mentioning that some of the early seminal studies were based on V1V3 amplicon sequencing using the Roche 454 platform (Perez-Carrasco et al., 2021), which allows longer reads. Therefore, up to now library preparation kits and sequencing platforms have heavily influenced the choice of 16S rRNA hypervariable regions used

in urinary microbiota profiling studies. Consequently, studies that provide evidence for a more informed choice are urgent.

In this study, we tested the performance of six commonly used 16S rRNA primer sets, spanning all nine hypervariable regions, for microbiota profiling of 22 urine samples collected from male volunteers by transurethral catheterization. We show that V1V2 amplicon sequencing is more suitable for male urinary microbiota profiling. We found that V1V2 provides the greatest taxonomic and ASV richness, which translates into a higher number of exclusively detected genera. This result is likely attributed to V1V2 having a higher taxonomic resolution for assessing the taxa commonly present in male urine samples.

We also evaluated combinations of pairs of non-overlapping amplicons, from which we observed only marginal gains in taxonomic richness in comparison with single amplicons. Combining all six amplicons leads to a substantial increase in taxonomic richness at species level, but with little impact on the overall taxonomic compositions, indicating these gains are largely due to low-abundant taxa. Therefore, they may not compensate for the higher costs of sequencing multi-amplicon libraries. Moreover, as amplicon combinations cannot be reconstructed as single sequences, the eventual equivocal association between amplicons may have caused inflation of taxonomic richness by false-positive taxa in Sidle-reconstructed datasets.

We observed huge discrepancies between amplicon-specific datasets when evaluating bacterial compositions by taxa relative abundances. This is mainly because some amplicons presented lower taxonomic resolution for profiling specific clades. Low taxonomic resolution may impact community-wide metrics and preclude the identification of associations between taxa and covariates. Furthermore, low taxonomic resolution may also drastically impact beta-diversity metrics that do not take phylogenetic information into account (e.g., Bray-Curtis).

Amplicon-specific datasets also differed in the set of taxa detected. V1V2 profiling minimized the number of undetected genera, but because all other datasets possessed exclusively detected genera, we conclude that missing a fraction of the urine bacterial richness is inevitable with 16S rRNA amplicon sequencing. Still, low relative abundance taxa drive these observed differences so that analyses will not be harshly influenced by this limitation, except when evaluating beta-diversity with metrics that do not take bacterial evenness into account (e.g., Jaccard).

Focusing on species cultured from the female urobiome and using an in silico approach with 16S rRNA sequences retrieved from the SILVA database, Hoffman et al. (2021) showed that taxonomic assignment algorithms, 16S rRNA databases, and the choice of 16S rRNA hypervariable regions influence the taxonomic profiles of female urine. Although they used an in silico approach and did not evaluate the same set of hypervariable regions, in agreement with our results, they show that the use of either V1V3 or V2V3 should be preferred for urobiome profiling due to higher taxonomic resolution. Noteworthy, they also showed that V1 and V2 hypervariable regions present the highest sequence entropy for the bacteria found in the female urinary microbiota.

In this study, removal of contaminants was a key step, since laboratory and reagent contaminants disproportionately affect the microbiota profiling of low bacterial biomass samples (Karstens et al., 2018). However, the method used for contaminant removal has limitations. Since we had low statistical power to detect contaminants exclusively using sequencing data, we validated our findings using information available in the literature. This is questionable because most urobiome studies available did not use strategies to control for contaminants (Cumpanas et al., 2020), therefore a previous description of a taxon in human urobiomes does not imply it is a true urinary tract-resident microbe. On the other hand, some lists of known reagent and laboratory contaminants are available in the literature [e.g., Salter et al. (2014)], but many of the taxa included in such lists are known to be present in human microbiotas. Obviously, these disputes are more frequent when studying less characterized environments. For instance, the genus *Variovorax*, which dominated a few samples in our study, is described as a contaminant by Salter et al. (2014). At the same time, in a contaminant-controlled study, a *Variovorax* strain was identified in the urethra of a non-chlamydial non-gonococcal urethritis patient (Riemersma et al., 2003).

Another important limitation of our study is the lack of information on what is the true taxonomic composition of the samples we analyzed. Because this is virtually impossible to infer completely, some studies comparing 16S hypervariable regions have determined the best region by comparing sequencing results with PCR quantification of key taxa (Cabral et al., 2017; Kameoka et al., 2021). Here, due to the lack of an internal reference, we also compared amplicon-specific datasets to a bioinformatic reconstruction of the microbial community present in the urine samples using the full set of 16S hypervariable regions. Further studies with experimentally validated references will be needed to confirm our findings.

Because genitalia and the urinary tract contain distinct bacterial communities (Gottschick et al., 2017), an important variable in urobiome studies is the choice of the sampling method (Brubaker et al., 2021a). Many urobiome studies evaluate voided urine samples (Cumpanas et al., 2020), which may contain bacteria from the urethra and genital skin, such that voided urine samples represent the whole urogenital tract. In this study, we evaluated urine samples collected *via* transurethral catheterization, which reduces the presence of distal urinary tract contaminants compared to voided urine urinary tract contaminants compared to voided urine (Dong et al., 2011; Southworth et al., 2019; Chen et al., 2020; Dornbier et al., 2020). This sampling method, similarly to suprapubic aspiration, allows the specific characterization of the urinary bladder microbiota (Wolfe and Brubaker, 2019). Even though this was a fundamental consideration to avoid cross-site contamination, further studies will be necessary to evaluate whether our results extend to voided urine specimens. Likewise, since we included only male volunteers in this study, further studies including urine samples from females are desired to test whether our results can be extrapolated to the female urobiome.

In conclusion, similarly to other reports of primer bias in microbiota studies, we provided evidence that V1V2 is the most suitable 16S rRNA amplicon for the characterization of catheterized urine samples microbiotas from males. To our knowledge, this is the first study to address this question by systematically analyzing all 16S hypervariable regions. This is true not only for catheter-derived urine samples, but actually for any kind of urine sample. Despite our limited sample size, which may not fully represent male urinary microbiotas, we believe that our results might help other researchers make an informed decision about which 16S rRNA hypervariable regions to use for male urobiome analysis.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The name of the repository and accession number can be found below: European Nucleotide Archive (ENA) at EMBL-EBI; PRJEB49145.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Hospital Sírio-Libanês (#HSL 2018-72). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceptualization and study design: VH, AM, DB, DJ, MA, and AC. Volunteer recruitment and clinical evaluation: AM, DB, DJ, and MA. Samples preparation and sequencing: VH and LI. Bioinformatics and statistical analyses: VH. Writing original draft: VH and AC. Reviewing and editing the manuscript: PA, FB, and AM. Supervision: AC. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.862338/full#supplementary-material

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The Urobiomes of Adult Women With Various Lower Urinary Tract Symptoms Status Differ: A Re-Analysis

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The discovery of the urinary microbiome (urobiome) has created opportunities for urinary health researchers who study a wide variety of human health conditions. This manuscript describes an analysis of catheterized urine samples obtained from 1,004 urobiome study participants with the goal of identifying the most abundant and/or prevalent (common) taxa in five clinically relevant cohorts: unaffected adult women (n=346, 34.6%), urgency urinary incontinence (UUI) (n=255, 25.5%), stress urinary incontinence (SUI) (n=50, 5.0%), urinary tract infection (UTI) (n=304, 30.4%), and interstitial cystitis/painful bladder syndrome (IC/PBS) (n=49, 4.9%). Urine was collected *via* transurethral catheter and assessed for microbes with the Expanded Quantitative Urine Culture (EQUC) technique. For this combined analytic cohort, the mean age was 59 ± 16; most were Caucasian (n=704, 70.2%), Black (n=137, 13.7%), or Hispanic (n=130, 13.0%), and the mean BMI was 30.4 ± 7.7. Whereas many control or IC/PBS cohort members were EQUC-negative (42.4% and 39.8%, respectively), members of the other 3 cohorts were extremely likely to have detectable microbes. The detected urobiomes of the controls and IC/PBS did not differ by alpha diversity or genus level composition and differed by only a few species. The other 3 cohorts differed significantly from the controls. As expected, *Escherichia* was both prevalent and highly abundant in the UTI cohort, but other taxa also were prevalent at more moderate abundances, including members of the genera *Lactobacillus, Streptococcus, Staphylococcus, Corynebacterium*, *Actinomyces*, and *Aerococcus.* Members of these genera were also prevalent and highly abundant in members of the UUI cohort, especially *Streptococcus anginosus*. Intriguingly, these taxa were also detected in controls but at vastly lower levels of both prevalence and abundance, suggesting the possibility that UUI-associated symptoms could be the result of an overabundance of typical urobiome constituents. Finally, prevalence and abundance of microbes in the SUI cohort were intermediate to those of the UUI and control cohorts.

These observations can inform the next decade of urobiome research, with the goal of clarifying the mechanisms of urobiome community composition and function. There is tremendous potential to improve diagnosis, evaluation and treatment for individuals affected with a wide variety of urinary tract disorders.

Keywords: urinary microbiome, lower urinary symptom, enhanced culture, reanalysed data, women's health

INTRODUCTION

The discovery of the urinary microbiome has created opportunities for urinary health researchers in a wide variety of human health conditions. The first decade of urobiome studies have revealed clear associations between various microbes and various urinary symptoms and diagnoses. Several species have been associated with urgency urinary incontinence, including *Actinotignum* (previously *Actinobaculum*) *schaalii*, *Streptococcus anginosus*, and *Aerococcus urinae* (Pearce et al., 2014). Some evidence supports the hypothesis that *A. urinae* is also associated with urinary tract infection; since standard urine culture often misses *A. urinae* (Price et al., 2016), this and other evidence induces doubts concerning diagnoses of exclusion, such as UUI. Some women with lower urinary tract symptoms have urobiomes predominated by supposedly beneficial taxa, especially *Lactobacillus* species (Pearce et al., 2014; Pearce et al., 2015; Thomas-White et al., 2018). Finally, some evidence exists associating pre-treatment urobiome status with response to medical UUI treatment with an anticholinergic medication (Thomas-White et al., 2016) and response to surgical treatment with midurethral sling for mixed urinary incontinence (Richter et al., 2022).

However, most early studies were small with sample sizes in the few dozens and were designed to only identify associations. As we begin the second decade of urobiome research, studies with increased samples sizes will be needed to verify and test these associations and their underlying mechanisms. To inform those efforts, this manuscript describes an analysis of catheterized urine samples obtained from 1,004 urobiome study participants (both unpublished and previously published). Our goal was to characterize the urobiome of women with lower urinary tract symptoms and identify the most abundant and/or prevalent (common) taxa in five clinically relevant cohorts. These cohorts were unaffected adult women, as well as those affected by common lower urinary tract conditions, including urinary tract infection (UTI), interstitial cystitis/painful bladder syndrome (IC/PBS), and two common forms of urinary incontinence: urgency urinary incontinence (UUI) and stress urinary incontinence (SUI). This descriptive analysis may inform future prospective work that incorporates knowledge of the urobiome composition in diagnosing and treating women presenting with symptoms.

METHODS

Recruitment of Participants

The urobiome of 1004 participants previously reported in eight published studies were reanalyzed using five cohorts of clinical interest: UTI (n=304, 30.4%), UUI (n=255, 25.5%), SUI (n=50, 5.0%), IC/PBS (n=49, 4.9%), and controls (n=346, 34.6%) without these lower urinary tract symptoms. The inclusion criteria for each cohort were based on predominant presenting symptoms/diagnoses as assessed by the relevant validated questionnaire (see original publications). These participants were pooled from 8 separate, IRB-approved studies with identical baseline assessment and sample collection procedures. 921 of these participants were at least partially described previously (**Supplemental Table 1**). These studies were supported by NIDDK (R01DK104718-01A1, R56DK104718-01, R21DK097435, and P20DK108268), a PFD Research Grant and a grant from the Falk Foundation (LU202567). The funders played no part in the design or conduct of the study.

Urine Collection and Analysis

Urine was collected aseptically *via* transurethral catheter, according to standard clinical protocols, and placed in a BD Vacutainer Plus C&S preservative tube. To detect microbes, Expanded Quantitative Urine Culture (EQUC) was used, as described (Hilt et al., 2014). Briefly, 0.1 mL of urine was spread quantitatively onto BAP, Chocolate and Colistin, Naladixic Acid (CNA) agars (BD BBL™ Prepared Plated Media), then incubated in 5% CO_2 (35°C for 48 hours). A second set of BAPs were inoculated with 0.1 mL of urine and incubated in room atmosphere at 35°C and 30°C for 48 hours, respectively. In addition, 0.1 mL of urine was inoculated onto each of two CDC Anaerobe 5% sheep blood agar (ABAP) plates (BD BBL™ Prepared Plated Media) and incubated in either a Campy gas mixture (5% O_2, 10% CO_2, 85% N) or in anaerobic conditions at 35° C for 48 hours. The detection level was 10 CFU/mL, represented by 1 colony of growth on any of the plates. Each morphologically distinct colony type was isolated on a different plate of the same media to prepare a pure culture that was used for microbe identification. Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS) with the MALDI Biotyper 3.0 software (Bruker Daltonics, Billerica, MA) was used to identify the bacterial isolates.

Statistical Analysis

Generalized linear models were used to calculate age-adjusted estimates of microbial abundance, prevalence, and diversity for each cohort. Omnibus tests for cohort differences were assessed for each model, and group comparisons were reported as significant when Sidak-corrected p-values were less than 0.05. Alpha diversity measures included the unique number of genera (richness), Pielou's index (evenness), the Shannon index (richness and evenness), and Simpson index (richness and abundance). Each alpha diversity measure was regressed on age and cohort in a separate general linear model with normal distribution and identity link. Alpha

diversity measures were also correlated with age and body mass index, and Spearman's rho with 95% confidence intervals were estimated for each cohort. For each genus, abundance (mean CFU/mL) was modeled as a negative binomial distributed variable and regressed on cohort and age. Prior to modeling, mean CFU/mL for any taxon was calculated by dividing the total number of CFU/mL by the number of plates with growth for that taxon. The prevalence of each genus or species was defined as the proportion of individuals with any CFU/mL detected, and logistic regression analyses were conducted to estimate the adjusted mean prevalence for each cohort. The probability of no growth was modeled in a separate age-adjusted logistic regression analysis. SAS version 9.4 was used for statistical modeling, and microbiome diversity measure calculations and data visualization were performed using R version 4.0.3.

RESULTS

The demographics of each study-specific cohort were described within the original studies. For this combined analytic cohort, the mean age was 59 ± 16, most were Caucasian (n=704, 70.2%), Black (n=137, 13.7%), or Hispanic (n=130, 13.0%), and mean BMI was 30.4 ± 7.7. The UTI and UUI cohorts were 10-15 years older on average than the other 3 cohorts. Participants in the control and IC/PBS cohorts were most likely to have no growth by EQUC: 42.4% and 39.8% of each cohort, respectively. In contrast, growth was not detected in only 5.6%, 7.9%, and 0.5% of the UTI, UUI and SUI cohorts, respectively (**Table 1**).

After adjusting for age, the detected urobiomes of the UTI and control cohorts differed in the number of genera (a measure of richness) and Simpson's Index (which measures richness and abundance) but not Peilou's evenness or Shannon's Index (which measures both richness and evenness) (**Table 2**). The UUI and controls differed in number of genera, Shannon's Index, and Peilou's evenness, but not Simpson's Index. The SUI and control cohorts differed in all 4 measures of alpha diversity, whereas the IC/PBS and control cohorts were similar. The UTI and UUI cohorts differed in all 4 measures, while the 2 incontinent cohorts (UUI and SUI) differed in the number of species and Shannon's Index (**Table 2**). Correlations of age and body mass index with diversity indices were small to moderate across cohorts (**Supplemental Table 2**). Older age was associated with higher diversity indices in the UUI and SUI cohorts and lower diversity indices for UTI. Higher BMI was associated with a greater number of genera detected across cohorts, and higher Shannon and Simpson indices in the UTI cohort.

Of the detected genera, 20 were present in at least 2% of participants and investigated in models of prevalence by cohort (**Figure 1** and **Table 3**). *Escherichia* was more prevalent in the UTI cohort (48.5%) than all others, but several other genera were detected in more than 10% of the UTI members, including *Lactobacillus* (39.7%), *Streptococcus* (27.8%), *Staphylococcus* (16.5%), *Corynebacterium* (14.9%), *Gardnerella* (13.2%), and *Actinomyces* (11.5%). More genera were commonly detected in the UUI cohort, with over 20% adjusted prevalence of each of the most prevalent genera in the UTI cohort plus *Enterococcus*. Among those with SUI, *Lactobacillus, Streptococcus*, and *Staphylococcus* were the genera most often detected. Prevalence was generally lower in the IC/PBS and control cohorts across all genera, with *Lactobacillus* and *Streptococcus* the most common of those detected.

Nine genera were both prevalent in at least 10% of participants across cohorts and exhibited significant cohort differences in abundance: *Lactobacillus, Streptococcus, Escherichia, Staphylococcus, Corynebacterium, Actinomyces, Aerococcus, Gardnerella*, and *Enterococcus* ($p<0.05$ for all omnibus tests of cohort differences) (**Figure 2** and **Table 4**). *Corynebacterium*, *Lactobacillus, Streptococcus, Actinomyces* and *Gardnerella* were significantly more abundant (generally 2 orders of magnitude) in participants with UUI than those in the non-UTI cohorts; these

TABLE 1 | Participant characteristics.

	UTI	UUI	SUI	IC/PBS	Control
N	304	255	50	49	346
Age, mean (SD) [n=1001]	66 (16)	65 (12)	54 (14)	51 (16)	50 (14)
Body mass index (kg/m²), mean (SD) [n=914]	29.8 (7.2)	31.8 (8.5)	30.2 (6.9)	–	29.9 (7.5)
Race/ethnicity, n (%) [n=1003]					
Non-Hispanic White	224 (73.7)	182 (71.4)	36 (72.0)	33 (67.3)	229 (66.4)
Non-Hispanic Black	34 (11.2)	42 (16.5)	3 (6.0)	1 (2.0)	57 (16.5)
Hispanic	39 (12.8)	23 (9.1)	8 (16.0)	13 (26.5)	47 (13.6)
Other	7 (2.3)	8 (3.1)	3 (6.0)	2 (4.1)	12 (3.5)
EQUC-negative (%)	5.6	7.9	0.5	39.8	42.4

TABLE 2 | Age-adjusted diversity indices.

	UTI	UUI	SUI	IC/PBS	Control
Number of genera	2.3 ± 0.1^{be}	5.1 ± 0.1^{acde}	2.3 ± 0.3^{be}	1.6 ± 0.3^{b}	1.3 ± 0.1^{abc}
Pielou's evenness*	0.22 ± 0.02^{bc}	0.51 ± 0.02^{ae}	0.48 ± 0.05^{ae}	0.35 ± 0.06	0.23 ± 0.02^{bc}
Shannon index	0.16 ± 0.03^{bc}	0.77 ± 0.03^{acde}	0.39 ± 0.06^{abe}	0.33 ± 0.06^{b}	0.19 ± 0.02^{bc}
Simpson index	0.17 ± 0.02^{bcde}	0.47 ± 0.02^{a}	0.34 ± 0.05^{ade}	0.58 ± 0.05^{ac}	0.54 ± 0.02^{ac}

*[a]Different from UTI; [b]different from UUI; [c]different from SUI; [d]different from IC/PBS; [e]different from Control. *calculated among those with at least two genera.*

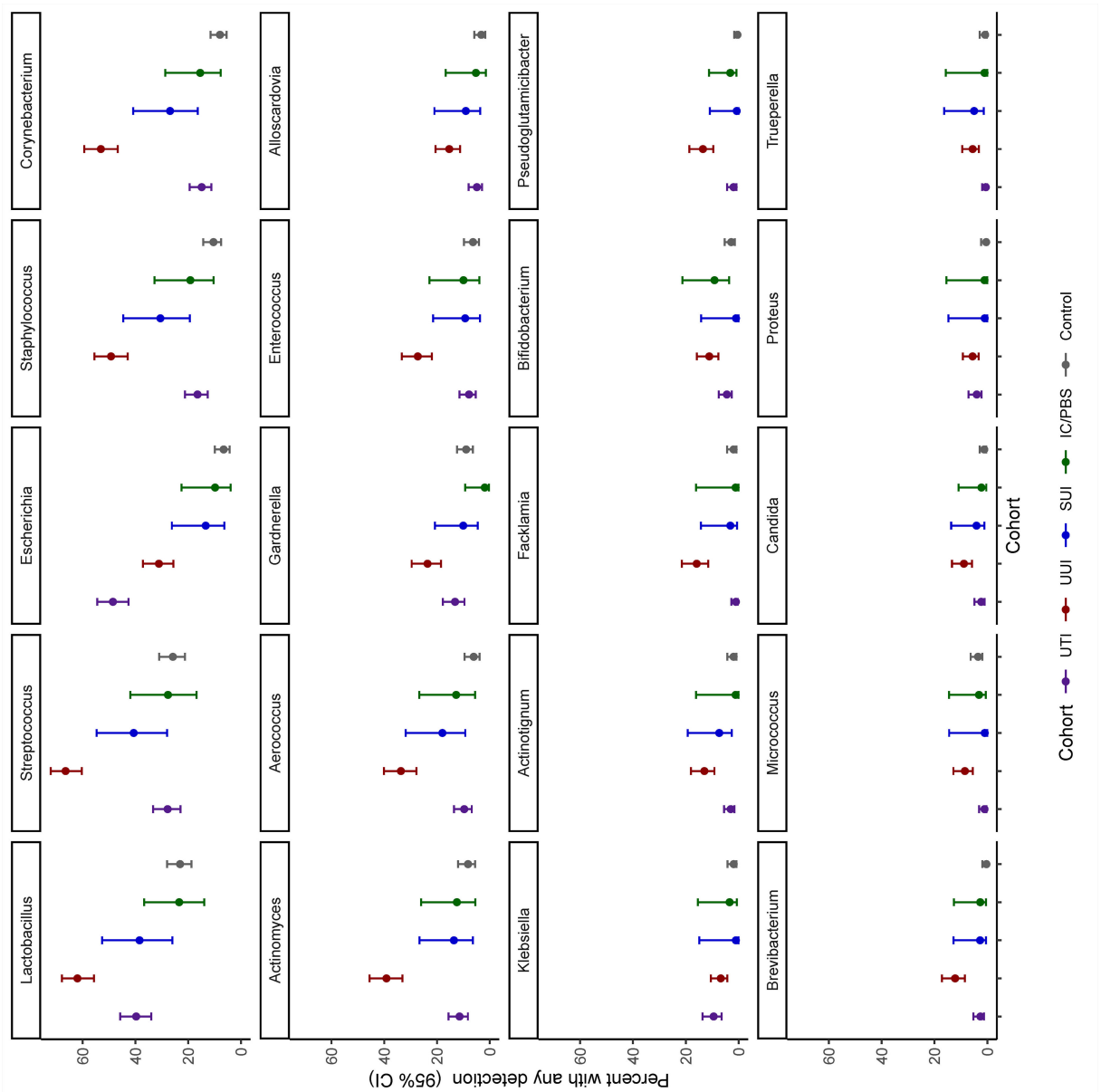

FIGURE 1 | Age adjusted prevalence (%) with 95% confidence interval by cohort.

TABLE 3 | Age adjusted prevalence (%) ± standard error.

	UTI	UUI	SUI	IC/PBS	Control
Lactobacillus	39.7 + 3.0[be]	62.0 + 3.1[acde]	38.4 + 6.9[b]	23.4 + 5.9[b]	23.1 + 2.4[ab]
Streptococcus	27.8 + 2.7[b]	66.5 + 3.0[acde]	40.7 + 7.0[b]	27.7 + 6.5[b]	25.8 + 2.5[b]
Escherichia	48.5 + 3.0[bcde]	31.1 + 3.0[ae]	13.4 + 4.9[a]	9.8 + 4.4[a]	6.6 + 1.4[ab]
Staphylococcus	16.5 + 2.2[b]	49.2 + 3.2[ade]	30.5 + 6.5[e]	19.2 + 5.7[b]	10.4 + 1.7[bc]
Corynebacterium	14.9 + 2.1[b]	53.1 + 3.2[acde]	26.9 + 6.3[be]	15.5 + 5.3[b]	8.0 + 1.5[bc]
Actinomyces	11.5 + 1.9[b]	39.2 + 3.2[acde]	13.6 + 5.0[b]	12.5 + 5.0[b]	8.3 + 1.6[b]
Aerococcus	9.7 + 1.7[b]	33.7 + 3.1[ae]	18.0 + 5.7	12.8 + 5.2	6.1 + 1.4[b]
Gardnerella	13.2 + 2.1[b]	23.6 + 2.8[ade]	10.1 + 3.9	1.9 + 1.6[b]	9.0 + 1.5[b]
Enterococcus	7.9 + 1.6[b]	27.3 + 2.9[ae]	9.3 + 4.2	10.0 + 4.5	6.4 + 1.4[b]
Alloscardovia	4.9 + 1.3[b]	15.4 + 2.4[ae]	9.1 + 4.1	5.3 + 3.3	3.2 + 1.0[b]
Klebsiella	9.5 + 1.8[e]	6.8 + 1.6	1.0 + 1.5	3.4 + 2.8	1.9 + 0.8[a]
Actinotignum	3.0 + 0.9[b]	13.0 + 2.2[ae]	7.4 + 3.8	1.1 + 1.6	1.9 + 0.8[b]
Facklamia	1.0 + 0.5[b]	15.9 + 2.5[ae]	3.1 + 2.5	1.2 + 1.6	1.9 + 0.8[b]
Bifidobacterium	4.4 + 1.2[b]	11.1 + 2.0[ae]	1.0 + 1.4	9.2 + 4.2	2.8 + 0.9[b]
Pseudoglutamicibacter	1.9 + 0.8[b]	13.5 + 2.3[ae]	0.7 + 1.0	3.2 + 2.1	0.5 + 0.3[b]
Brevibacterium	2.7 + 1.0[b]	12.2 + 2.2[ae]	2.8 + 2.3	2.7 + 2.2	0.4 + 0.3[b]
Micrococcus	1.1 + 0.6[b]	8.6 + 1.8[a]	1.0 + 1.4	3.2 + 2.6	3.6 + 1.1
Candida	2.4 + 0.9[b]	8.9 + 1.9[ae]	4.1 + 2.6	2.3 + 1.9	1.2 + 0.5[b]
Proteus	4.0 + 1.2	5.6 + 1.5[e]	1.0 + 1.5	1.1 + 1.6	0.5 + 0.4[b]
Trueperella	0.6 + 0.4[b]	5.6 + 1.6[a]	5.1 + 3.2	1.1 + 1.6	0.9 + 0.5
No growth	5.6 + 1.4[de]	7.9 + 1.7[de]	10.5 + 4.3[de]	39.8 + 7.1[abc]	42.2 + 2.9[abc]

[a]*Different from UTI;* [b]*different from UUI;* [c]*different from SUI;* [d]*different from IC/PBS;* [e]*different from Control.*

genera were more abundant in the UUI than the UTI cohort, except that the difference was generally 1 order of magnitude. *Corynebacterium* and *Actinomyces* were the notable exceptions, being 2 orders of magnitude more abundant in the UUI cohort than the UTI cohort. *Aerococcus* was significantly more abundant in the UUI cohort compared to the non-UTI cohorts. *Escherichia* was significantly more abundant in those with UTI compared to UUI, SUI, IC/PBS, and controls, but it was considerably more abundant in UUI and controls relative to SUI and IC/PBS. *Staphylococcus* was more abundant in the UTI and UUI cohorts compared to the other cohorts.

Of the nine prevalent genera with differential abundances, 6 included multiple species: *Lactobacillus, Streptococcus, Staphylococcus, Corynebacterium, Actinomyces*, and *Aerococcus* (**Table 5**). Most species in these genera were differentially more prevalent in the UUI cohort than the controls: 4 of 5 *Lactobacillus* species, 2 of 6 *Streptococcus* species, 5 of 7 *Staphylococcus* species, 9 of 9 *Corynebacterium* species, 2 of 3 *Actinomyces* species, and both *Aerococcus* species. *Streptococcus anginosus* merits special mention; it was more prevalent in the UUI cohort relative to all the other cohorts, being detected in 49% of that cohort. Another noteworthy species was *Staphylococcus epidermidis*, which was more prevalent in the UUI, SUI and IC/PBS cohorts relative to the controls.

Of all those species, only 9 were differentially abundant (*Streptococcus anginosus, Lactobacillus gasseri, Aerococcus urinae, Staphylococcus epidermidis, Lactobacillus iners, Corynebacterium coyleae, Actinomyces neuii, Lactobacillus jensenii*, and *Corynebacterium amycolatum*) (**Table 6**). Relative to the controls, 6 were more abundant in the UTI cohort, whereas all 9 were significantly more abundant in the UUI cohort. Again, *S. anginosus* merits notice; it was almost 10 and 100 times more abundant in the UTI and UUI cohorts, respectively. Whereas all 3 of the *Lactobacillus* species were more abundant in the UUI cohort, only *L. gasseri* was more abundant in the UTI cohort. *A. urinae* was more abundant in the UTI, UTI and IC/PBS cohorts, *S. epidermidis* was more abundant in the UTI and UUI cohorts, and *A. neuii* was more abundant in the UUI cohort. Whereas *C. amycolatum* was more abundant in the UTI, UUI, and IC/PBS cohorts, *C. coyleae* was more abundant in the UUI cohort but less abundant in the UTI cohort.

DISCUSSION

Amongst these five cohorts of clinical interest, we detected group differences in urobiome characteristics. However, despite these group differences, the cohorts have between-group overlap that precludes accurate predictions of diagnostic categorization. In addition, within group variation suggests that a range of urobiome characteristics cannot be associated with a single diagnostic cohort. This is consistent with the evolving idea that many lower urinary tract symptoms have heterogenous etiologies and/or mechanisms. Nonetheless, the trends observed can be used to inform the second decade of urobiome research, with the goal of clarifying the mechanisms of urobiome community function and membership. We are hopeful that future, well-designed longitudinal research will contribute valuable insights.

The etiology of painful bladder syndrome and interstitial cystitis continues to challenge clinicians and researchers who hope to find a way to ease the chronic suffering of affected patients. Although many hoped to find a single causative microbe, the "single microbe" hope has not been supported by research to date. Women with IC/PBS were just as likely to be EQUC-negative as healthy controls. Of those with detectable urobiomes, there was no difference in alpha diversity or

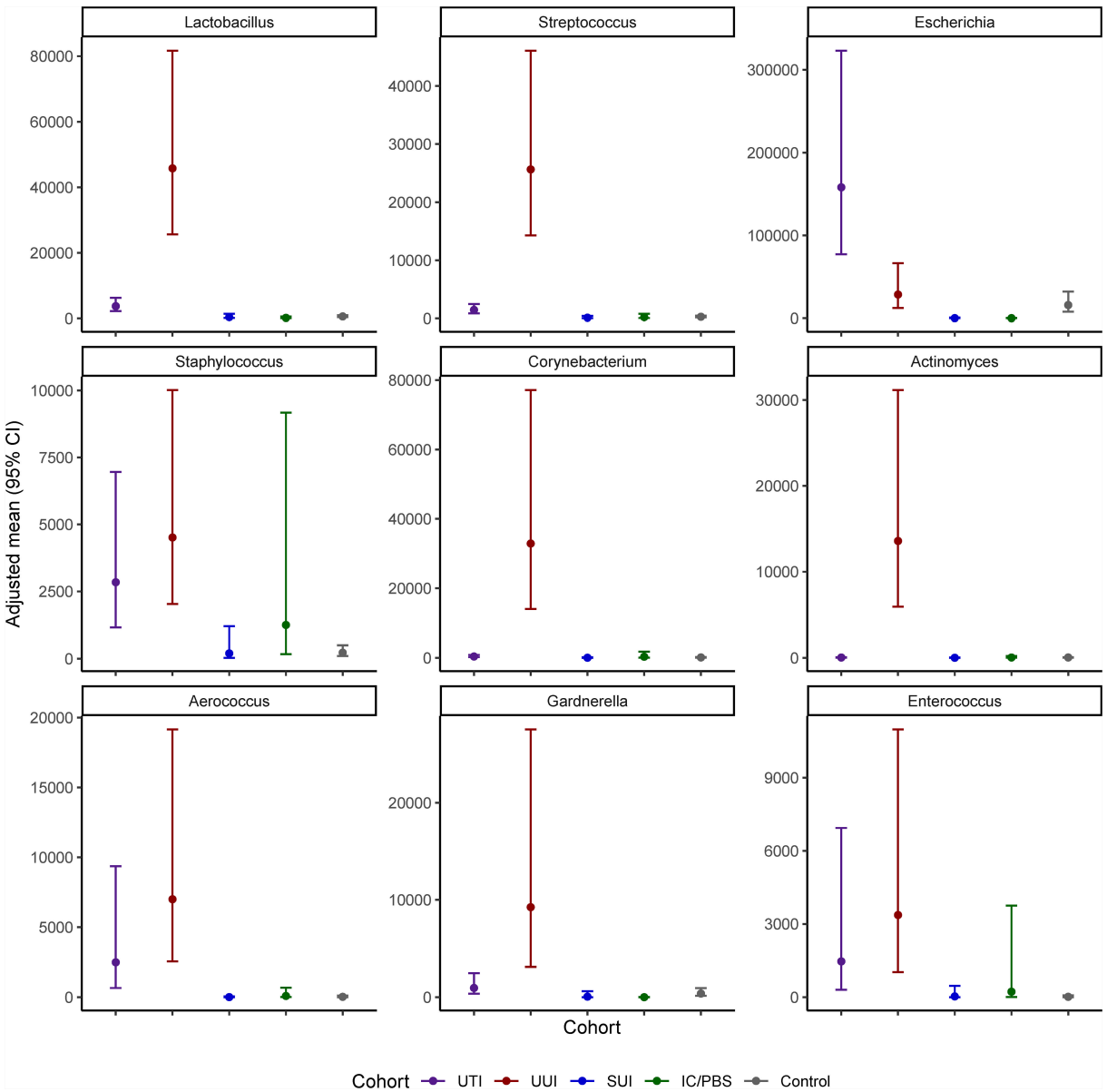

FIGURE 2 | Age adjusted mean abundance (CFU/mL) with 95% confidence interval by cohort.

TABLE 4 | Age adjusted mean (CFU/mL) ± standard error.

	UTI	UUI	SUI	IC/PBS	Control
Lactobacillus	3703 + 999^{bcde}	45774 + 13531^{acde}	379 + 252^{ab}	149 + 100 ^{ab}	580 + 148 ^{ab}
Streptococcus	1458 + 396^{bce}	25640 + 7671^{acde}	118 + 79^{ab}	217 + 145^{b}	279 + 72^{ab}
Escherichia	158063 + 57687^{bcde}	28559 + 12304^{acd}	137 + 124^{abe}	57 + 53^{abe}	15871 + 5706^{acd}
Staphylococcus	2843 + 1299^{e}	4513 + 1836^{ce}	197 + 182^{b}	1250 + 1271	223 + 90^{ab}
Corynebacterium	388 + 141^{bc}	32892 + 14307^{acde}	25 + 22^{ab}	317 + 279^{b}	105 + 35^{b}
Actinomyces	32 + 12^{b}	13587 + 5756^{acde}	5 + 5^{b}	32 + 30^{b}	38 + 13^{b}
Aerococcus	2484 + 1682^{ce}	6998 + 3595^{cde}	8 + 8^{ab}	90 + 93^{b}	26 + 21^{ab}
Gardnerella	953 + 463^{bd}	9251 + 5152^{acde}	59 + 71^{b}	2 + 2^{abe}	378 + 176^{bd}
Enterococcus	1463 + 1162^{e}	3367 + 2031^{ce}	35 + 47^{b}	228 + 326	19 + 14^{ab}

[a]Different from UTI; [b]different from UUI; [c]different from SUI; [d]different from IC/PBS; [e]different from Control.

TABLE 5 | Age adjusted prevalence (%) ± standard error.

	UTI	UUI	SUI	IC/PBS	Control
Lactobacillus					
crispatus	8.1 ± 1.6	15.1 ± 2.4[e]	10.3 ± 4.9	4.9 ± 2.7	5.7 ± 1.2[b]
gasseri	14.5 ± 2.1[b]	29.6 ± 3.6[ae]	12.9 ± 4.8	11.2 ± 4.6	9.6 ± 1.7[b]
iners	10.9 ± 1.9[b]	21.4 ± 2.8[ae]	15.3 ± 4.8	8.7 ± 3.4	6.8 ± 1.3[b]
jensenii	9.6 ± 1.8	18.0 ± 2.5[e]	14.2 ± 4.7	3.7 ± 2.3	6.0 ± 1.2[b]
rhamnosus	2.4 ± 3.9	3.2 ± 1.1	1.1 ± 1.5	1.2 ± 1.6	0.5 ± 3.4
Streptococcus					
agalactiae	8.7 ± 1.7	13.1 ± 2.2[e]	10.1 ± 4.2	9.8 ± 4.1	4.7 ± 1.1[b]
anginosus	16.4 ± 2.2[b]	49.3 ± 3.2[acde]	26.4 ± 6.2[be]	10.9 ± 4.5[b]	11.4 ± 1.8[bc]
mitis	3.7 ± 1.1[b]	9.9 ± 1.9[a]	7.0 ± 3.6	3.1 ± 2.5	4.6 ± 1.2
oralis	2.3 ± 6.8[bd]	8.8 ± 1.8[a]	7.3 ± 3.8	12.3 ± 5.5[a]	4.8 ± 1.3
parasanguinis	0.6 ± 5.4[bc]	4.8 ± 1.3[a]	7.4 ± 3.8[a]	5.8 ± 3.6	3.6 ± 1.1
salivarius	0.7 ± 0.4[b]	5.4 ± 1.4[a]	5.3 ± 3.3	1.1 ± 1.6	3.9 ± 1.1
Staphylococcus					
aureus	2.0 ± 7.8	4.0 ± 1.3	9.2 ± 4.2[e]	1.1 ± 1.5	1.4 ± 1.7[c]
capitis	1.2 ± 0.6	5.4 ± 1.5	3.0 ± 2.4	1.0 ± 1.4	1.3 ± 2.6
epidermidis	7.4 ± 1.5[b]	36.8 ± 3.2[ae]	18.8 ± 5.6[e]	17.2 ± 5.5[e]	4.3 ± 1.1[bcd]
haemolyticus	2.0 ± 2.8[b]	21.3 ± 2.8[ae]	5.1 ± 3.2	5.3 ± 3.3	1.0 ± 9.6[b]
hominis	2.8 ± 1.8[b]	19.3 ± 2.6[ae]	2.9 ± 2.4	6.9 ± 3.6	1.5 ± 7.7[b]
lugdunensis	2.1 ± 5.9[b]	8.4 ± 1.8[ae]	3.0 ± 2.4	7.2 ± 3.8[e]	0.4 ± 5.4[bd]
simulans	1.6 ± 9.7[b]	10.2 ± 2.2[ae]	5.2 ± 3.2	3.3 ± 2.6	1.1 ± 1.6[b]
Corynebacterium					
amycolatum	4.3 ± 1.2[b]	31.0 ± 3.1[acde]	3.0 ± 2.4[b]	7.2 ± 3.8[b]	1.9 ± 6.8[b]
aurimucosum	4.0 ± 1.2[b]	24.5 ± 2.9[ae]	10.4 ± 4.3[e]	4.7 ± 3.1	1.7 ± 7.7[bc]
coyleae	5.1 ± 1.3[b]	29.9 ± 3.3[ace]	8.8 ± 4.6[b]	11.1 ± 4.5	2.7 ± 8.9[b]
Imitans	2.9 ± 1.7[b]	12.1 ± 2.2[ae]	2.8 ± 2.3	4.7 ± 3.1	0.6 ± 8.4[b]
lipophile group	1.5 ± 9.7[b]	11.3 ± 2.1[ae]	7.3 ± 3.8[e]	3.4 ± 2.7	0.4 ± 9.4[bc]
Riegelii	0.7 ± 6.5[b]	11.7 ± 2.2[ae]	3.1 ± 2.5	3.3 ± 2.6	1.1 ± 2.6[b]
tuberculostearic	1.1 ± 2.6[b]	7.0 ± 1.7[ae]	1.0 ± 1.4	5.4 ± 3.3	0.7 ± 9.5[b]
tuscaniense	0.8 ± 6.5[b]	9.3 ± 2.9[ae]	1.0 ± 1.4	3.0 ± 2.5	0.4 ± 4.4[b]
urealyticum	0.3 ± 6.3[b]	6.2 ± 1.7[ae]	1.1 ± 1.5	1.2 ± 1.6	0.5 ± 3.4[b]
Actinomyces					
europaeus	1.3 ± 9.6[b]	6.3 ± 1.6[a]	1.1 ± 1.5	3.5 ± 2.8	1.5 ± 6.7[b]
neuii	6.2 ± 1.4[b]	23.4 ± 2.8[ae]	11.5 ± 4.7	5.6 ± 3.5	3.5 ± 1.1[b]
turicensis	2.7 ± 6.9[b]	13.7 ± 2.3[ae]	3.1 ± 2.5	5.8 ± 3.6	3.3 ± 1.1[b]
Aerococcus					
sanguinicola	2.8 ± 0.9[b]	12.4 ± 2.2[ae]	9.5 ± 4.3	1.1 ± 1.6	0.1 ± 7.2[b]
urinae	7.2 ± 1.4[b]	29.8 ± 3.1[ae]	15.7 ± 5.4	10.4 ± 4.8	4.4 ± 1.2[b]

[a]*Different from UTI;* [b]*different from UUI;* [c]*different from SUI;* [d]*different from IC/PBS;* [e]*different from Control.*

composition at the genus level, and only a few differences in either prevalence or abundance but not both at the species level. Although there are clear and profound clinical differences between these two cohorts (Jacobs et al., 2021), these findings suggest that there is not likely to be a major microbial etiology.

This analysis clearly demonstrates that, compared to the urobiomes of controls, richness was increased in the urobiome of the UUI, SUI and UTI cohorts, with the most richness found in the UUI cohort. While the UUI and SUI cohorts were richer, more even, and more abundant than the controls, the UUI cohort was richer than the SUI cohort and the UUI cohort was richer, more even and more abundant than the UTI cohort. Both forms of incontinence are chronic conditions, with likely longer term, chronic changes in the urobiome. Especially in women who

TABLE 6 | Age adjusted mean (CFU/mL) ± standard error

	UTI	UUI	SUI	IC/PBS	Control
Streptococcus anginosus	697 ± 258[bce]	10098 ± 3976[acde]	33 ± 29[ab]	131 ± 122[b]	84 ± 29[ab]
Lactobacillus gasseri	1034 ± 480[bce]	7782 ± 3674[acde]	10 ± 11[ab]	97 ± 104[b]	28 ± 12[ab]
Lactobacillus iners	384 ± 192[bd]	9655 ± 5150[acde]	155 ± 189[b]	8 ± 10[ab]	272 ± 125[b]
Lactobacillus jensenii	1105 ± 661	12724 ± 8098[cde]	23 ± 35[b]	18 ± 27[b]	224 ± 129[b]
Aerococcus urinae	564 ± 253[bce]	4127 ± 1853[acde]	4 ± 4[ab]	66 ± 68[be]	1 ± 1[abd]
Staphylococcus epidermidis	3489 ± 2488[ce]	4845 ± 2839[cde]	2 ± 3[ab]	51 ± 62[b]	20 ± 15[ab]
Actinomyces neuii	10 ± 5[b]	8872 ± 5010[acde]	4 ± 6[b]	3 ± 4[b]	31 ± 15[b]
Corynebacterium amycolatum	208 ± 118[bce]	12808 ± 8598[acde]	0 ± 0[abd]	30 ± 38[bce]	0 ± 0[abd]
Corynebacterium coyleae	7 ± 4[be]	5802 ± 3385[acde]	1 ± 2[be]	104 ± 132[b]	176 ± 84[abc]

[a]*Different from UTI;* [b]*different from UUI;* [c]*different from SUI;* [d]*different from IC/PBS;* [e]*different from Control.*

are seeking treatment for incontinence, the effect of evaluation and treatment may also affect the urobiome that was sampled in these patients. In contrast, UTI is typically episodic. While there is little research into microbial recovery following UTI, it is expected that the urobiome attempts to return to the "normal" state, ideally closer to the urobiome seen in the control cohort.

Within the UUI cohort, we detected multiple genera and species, many of which were prevalent and abundant. Many are of uncertain clinical significance and await further research to clarify the role of the individual microbe and its role in the function of the urobiome it inhabits. However, our findings that *Aerococcus urinae* is abundant in both UTI and UUI cohorts is not expected. Since *A. urinae* is often not detected on standard urine culture (Price et al., 2016), this finding supports the use of enhanced microbial detection methods in women with UUI, such as expanded culture techniques, to detect and appropriately treat this known uropathogen. Without appropriate detection, it is likely that symptomatic women would be deemed "infection free"; subsequent diagnosis and treatment would focus on a UUI diagnosis with the missed opportunity to treat a known uropathogen. *A. urinae*-associated UUI may be an important, treatable subset of UUI patients. This testable hypothesis should be addressed as a research priority as soon as feasible.

We observed the expected disproportionate prevalence and abundance of *Escherichia* in the UTI cohort. *Escherichia* was less prevalent in the UUI and control cohorts but at relatively high abundance when detected. Other genera differed across cohorts. For example, while *Lactobacillus* is generally considered to be beneficial, it is likely that the beneficial contributions are determined at the species level and possible that certain *Lactobacillus* species (and other presumed beneficial microbes) can be opportunistic uropathogens within a given urobiome. For example, our finding that *L. gasseri*, *L. iners*, and other presumed commensal/beneficial genera often detected in controls are more abundant in the UUI cohort may suggest the possibility that the urobiome of these individuals could be affected by overgrowth of commensal/beneficial genera, a suggestion made more than 30 years ago by Rosalind Maskell and her team (Wilkins et al., 1989). Other detected species are thought to be emerging uropathogens and have been reported to be associated with UUI (e.g., *S. anginosus* and *A. neuii* [recently renamed *Winkia neuii* (Nouioui et al., 2018)]).

This analysis benefits from multiple strengths, most notably the relatively large numbers once the five individual cohorts were pooled. Also, the urine samples were obtained by transurethral catheterization, increasing the likelihood that the urine is of bladder origin. Additionally, the use of EQUC ensures that living microbes are being assessed. Furthermore, all cohorts were characterized with well validated instruments for the specific lower urinary tract condition of interest. Finally, the results were adjusted for age, given the current evidence that age itself may affect microbial niches.

Limitations include knowledge that EQUC does not detect every bacterial taxon and is especially limited for strict anaerobes. It also does not detect viruses or most eukaryotic microbes. Thus, the list of genera and species is likely incomplete. Also, given the design of the initial studies, this analysis cannot provide insights into longitudinal changes for individuals or across cohorts. Future prospective longitudinal studies should consider change in the urobiome composition as relates to diagnosis, symptom severity, treatment options, response, and recurrence. Finally, current clinical diagnostic categories were adopted prior to discovery of the urobiome; these categories may require revision as research closes important knowledge gaps.

In conclusion, as we enter the second decade of urobiome research, there is tremendous potential to improve diagnosis, evaluation and treatment for individuals affected with a wide variety of urinary tract disorders. As the community of urobiome researchers expands, covering the entire urinary tract and exploring both benign and malignant disease, we expect that significant advances will rapidly occur. The next decade of research is also likely to benefit from less expensive microbiome technology, a larger pool of knowledgeable researchers who collaborate in multi-disciplinary teams, and an increased opportunity to acquire research funds.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: This is a reanalysis. The data are present in the original papers. Requests to access these datasets should be directed to awolfe@luc.edu.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Loyola University Chicago IRB. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CJ: Data Analysis, manuscript writing/editing. TH: Project development, data collection, data analysis, manuscript review. CG: Data collection, manuscript review. LB: Project development, manuscript writing/editing. AW: Project development, manuscript writing/editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.860408/full#supplementary-material

Supplemental Table 1 | Source of participants for this pooled analysis. (1) 111 peri-menopausal women with UUI prior to treatment with solifenacin or asymptomatic controls, (2) 154 women with UTI-like symptoms or asymptomatic controls, (3) 134 peri-menopausal asymptomatic controls, (4) 89 women with IC/PBS or asymptomatic controls, (5) 145 women with SUI, UUI, or asymptomatic controls, (6) 63 postmenopausal women with UUI prior to receipt of vaginal estrogen therapy, (7) 225 women with symptoms of UTI recruited to a trial evaluating the expanded urine culture protocol, and (8) 83 peri-menopausal women with UUI prior to oral treatment with mirabegron.

Supplemental Table 2 | Correlation of participant characteristics with diversity indices. Spearman's correlation coefficient calculated with 95% confidence interval separately for each cohort and each alpha diversity index.

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Gardnerella Exposures Alter Bladder Gene Expression and Augment Uropathogenic *Escherichia coli* Urinary Tract Infection in Mice

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The anaerobic actinobacterium *Gardnerella* was first isolated from the bladder by suprapubic aspiration more than 50 years ago. Since then, *Gardnerella* has been increasingly recognized as a common and often abundant member of the female urinary microbiome (urobiome). Some studies even suggest that the presence of *Gardnerella* is associated with urological disorders in women. We recently reported that inoculation of *Gardnerella* into the bladders of mice results in urothelial exfoliation. Here, we performed whole bladder RNA-seq in our mouse model to identify additional host pathways involved in the response to *Gardnerella* bladder exposure. The transcriptional response to *Gardnerella* reflected the urothelial turnover that is a consequence of exfoliation while also illustrating the activation of pathways involved in inflammation and immunity. Additional timed exposure experiments in mice provided further evidence of a potentially clinically relevant consequence of bladder exposure to *Gardnerella*—increased susceptibility to subsequent UTI caused by uropathogenic *Escherichia coli*. Together, these data provide a broader picture of the bladder's response to *Gardnerella* and lay the groundwork for future studies examining the impact of *Gardnerella* on bladder health.

Keywords: urobiome, bladder, dysbiosis, urothelium, bacterial vaginosis, urinary tract infection, RNA-seq

INTRODUCTION

Gardnerella comprises a genus of Gram-variable Actinobacteria that are frequently present in the microbiota of the female urogenital system. *Gardnerella vaginalis* has historically been regarded as a vaginal organism because it was first identified in vaginal fluid, where it was implicated as the causative agent in the prevalent condition clinically recognized as bacterial vaginosis (BV) (Leopold, 1953; Gardner and Dukes, 1954; Schwebke et al., 2014; Morrill et al., 2020). BV is a state of the vaginal microbiome that is composed of a polymicrobial mixture of anaerobic bacteria. Further studies over the past 50 years have revealed that *G. vaginalis* is frequently found among the vaginal microbiota outside the context of symptomatic BV (Krohn et al., 1989; Briselden and Hillier, 1990).

However, even in asymptomatic women, *Gardnerella* is more frequently present and is often the predominant organism in the context of a vaginal "community state type" (CST) that is composed of a polymicrobial mixture of anaerobic bacteria (Ravel et al., 2011). *G. vaginalis* has a complicated taxonomic history, originally being named *Haemophilus vaginalis* and then *Corynebacterium vaginale* (Gardner and Dukes, 1955; Zinnemann and Turner, 1963). Most recently, a split of the *Gardnerella* genus into 13 distinct species has been proposed (Vaneechoutte et al., 2019). Some strains that have been characterized and previously referred to in the literature as *G. vaginalis* would fall into a different species with the newly proposed nomenclature, including the strain we used in this study. For ease of understanding, here, we primarily use only the genus name *Gardnerella.*

Urine is the second most common source of isolation of *Gardnerella*, after the vagina. *Gardnerella* is a rare cause of symptomatic urinary tract infection (UTI), and it is more often detected in urine samples collected in studies aimed at profiling the composition of the urinary microbiome, or "urobiome" (Kline and Lewis, 2016). In the first report of isolation from the bladder in 1968, *Gardnerella* was cultured from 159/1000 suprapubic aspirates from healthy pregnant women (Mcfadyen and Eykyn, 1968). Subsequent culture-based studies isolated *Gardnerella* in bladder aspirates from women with and without current or prior urinary tract diseases (Birch et al., 1981; McDowall et al., 1981; McDonald et al., 1982; Fairley and Birch, 1983; Gilbert et al., 1986). In studies using modern 16S sequencing and expanded quantitative culture methods, *Gardnerella* has emerged as one of the most frequently isolated members of the female urobiome and is the dominant organism in many women (Hilt et al., 2014; Pearce et al., 2014; Pearce et al. 2015; Gottschick et al., 2017; Jacobs et al., 2017; Price et al., 2020). Like *Gardnerella*, a majority of other urobiome bacteria are members of genera historically regarded as vaginal organisms, such as *Lactobacillus*. Recent studies have found substantial overlap between the urinary and vaginal microbiomes present concurrently in the same woman (Komesu et al., 2019; Brown et al., 2021; Hugenholtz et al., 2022). Although the presence of *Gardnerella* in urine specimens could reflect periurethral or vaginal colonization, the fact that many studies have cultured *Gardnerella* from urine collected directly from the bladder by suprapubic aspiration or catheterization strongly suggests that *Gardnerella* gains access to the bladder, at least transiently, in some women.

Whether, or how, urobiome members such as *Gardnerella* stably colonize the bladder remains to be determined. We also know very little regarding how the bladder responds to bacterial exposures outside of the context of symptomatic UTI. This is important because there has been growing interest in the concept of manipulating the urobiome as a therapeutic strategy for a wide range of lower urinary tract conditions (Jung and Brubaker, 2019; Cole et al., 2021; Jones et al., 2021; Garofalo et al., 2022). Mouse models have proven valuable in advancing our understanding of bladder responses to established uropathogens, but animal models examining common urobiome bacteria are limited. We previously developed a mouse model of *Gardnerella* bladder exposure (Gilbert et al., 1986; O'Brien et al., 2020). We refer to this model as an "exposure" rather than an infection because *Gardnerella* is cleared from the urinary tract within 12 h. Even such a transient presence of *Gardnerella* in the urinary tract was sufficient to trigger apoptosis and exfoliation of the superficial bladder epithelial (urothelial) cells (Gilbert et al., 2017). Exfoliation is an innate host response known to occur during symptomatic UTI that is presumably aimed at helping eliminate bacteria from the bladder *via* shedding of infected epithelial cells (Mysorekar and Hultgren, 2006; Lin et al., 2015). Here, we further probed the host response to *Gardnerella* in the urinary tract by examining the bladder transcriptome using RNA-seq. Two successive exposures to *Gardnerella* activated genes and pathways in the bladder that are related to DNA damage, programmed cell death, cell differentiation, and proliferation, which are consistent with the processes of urothelial exfoliation and renewal. Additionally, *Gardnerella* exposure influenced gene sets related to immune and inflammatory responses. Finally, we demonstrate that preexposure to *Gardnerella* resulted in heightened bacterial loads upon subsequent uropathogenic *Escherichia coli* (UPEC) experimental UTI, promoting persistent UPEC bacteriuria and increased bladder tissue titers. These findings provide proof of concept that even transient *Gardnerella* bladder exposures affect the bladder mucosa in ways that can alter the course of UPEC UTI.

RESULTS

Effect of *Gardnerella* Exposures on the Bladder Transcriptome

We performed RNA-seq on whole bladders to identify host responses to *Gardnerella* exposures in naive mice. **Figure 1A** summarizes the experimental timeline. Our previous experiments demonstrated that *Gardnerella* is cleared from the mouse bladder within 12 h and that two exposures are required to elicit urothelial exfoliation (Gilbert et al., 1986). Presently, five female C57BL/6 mice were inoculated twice intravesically with *Gardnerella* strain JCP8151B. Four age-matched female C57BL/6 mice were inoculated twice with PBS in parallel (PBS) to serve as controls. Exposures were given 12 h apart, and bladders were collected 12 h after the second exposure.

RNA was extracted individually from each bladder and used for RNA-seq. A total of 305,893,605 RNA-seq reads were generated. Of these, 207,076,414 unique reads could be aligned to the *Mus musculus* reference genome. Further details of the RNA-seq reads are found in **Supplementary Table S1**. Differentially expressed genes (FDR adjusted $p < 0.05$, $\log_2 FC > 2$) and pathways were identified by comparing bladders in the *Gardnerella* group to PBS controls. At the individual gene level, *Gardnerella* exposure resulted in significantly increased expression of 38 genes and decreased expression of 11 genes relative to PBS controls (**Figure 1B**; **Table 1**). Gene set enrichment analyses, using both the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes

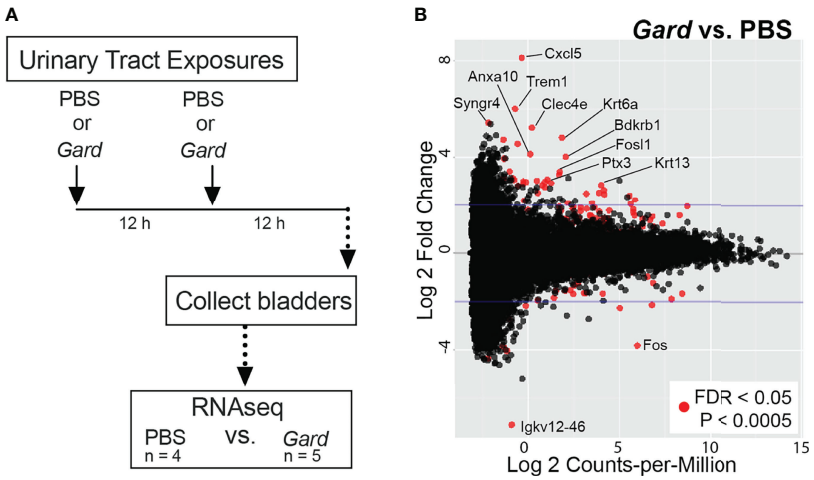

FIGURE 1 | *Gardnerella* exposures alter the bladder transcriptome. **(A)** Schematic of the urinary tract exposure model used for RNA-seq. Female C57BL/6 mice were given intravesical "urinary tract exposures" of either PBS or *Gardnerella via* transurethral catheterization. Each mouse received two exposures that were administered 12 h apart. Bladders were collected 12 h after the second exposure and processed for RNA-seq. *Gardnerella*-exposed bladders were compared to PBS-exposed control bladders to identify differentially expressed genes and for gene set enrichment analyses. **(B)** MA-plot indicates genes that had altered expression in *Gardnerella* compared to PBS bladders. Red dots denote genes that were significantly differentially expressed after false discovery rate (FDR) correction for multiple comparisons. Genes discussed in the text are indicated by name.

(KEGG) pathway databases, were performed on the full RNA-seq dataset to gain a broader perspective of the biological effects of *Gardnerella* exposures on the bladder (**Figure 2**). The GO term analyses detected significant enrichment ($p < 0.05$, $\log_2$FC > 2) of 153 GO biological processes and 16 GO molecular functions (**Supplementary Tables S2, S3**) in *Gardnerella* bladders compared to PBS. Sixteen KEGG pathways were significantly enriched ($p < 0.05$, $\log_2$FC > 2) (**Supplementary Table S4**). There were very few significantly de-enriched pathways across all three categories; only the GO molecular function "pheromone activity" and the "steroid hormone biosynthesis" KEGG pathway were moderately decreased. In addition to single direction changes (all genes in the pathway either went UP or DOWN), seven KEGG pathways were significantly dysregulated in ANY direction (some genes in the same pathway went up and others went down) (**Supplementary Table S4**). As a whole, the RNA-seq data pointed to two broad categories affected by *Gardnerella* exposure: (1) inflammation and immune response and (2) urothelial exfoliation and differentiation.

Inflammatory Pathways Are Upregulated After *Gardnerella* Exposures

As would be expected in a bacterial exposure model, most of the gene sets enriched in *Gardnerella*-exposed bladders were related to inflammatory responses. We previously reported higher levels of bladder IL-12p40 following *Gardnerella* exposure (Gilbert et al., 2017). The most highly upregulated GO biological processes (**Figure 2A**) were directly related to inflammation, including leukocyte migration and chemotaxis (dark blue dots) or the more general "response to…" terms such as "bacterium" or "external biotic stimulus" (light blue dots). This theme continued through the rest of the enriched GO biological process terms; 49 were related to immune and inflammatory processes involving cytokines, chemokines, and leukocytes (**Supplementary Table S2**), and 15 were relevant "response to…" and "defense response to…" terms such as "bacterium," "molecule of bacterial origin," "external biotic stimulus," and "other organism" (**Supplementary Table S2**). Activation of host inflammatory responses was also reflected in the upregulated GO molecular functions, with top hits indicating cytokine and chemokine activity and chemokine receptor binding (**Figure 2B**). Likewise, "cytokine–cytokine receptor interactions" and "chemokine signaling pathway" were among the upregulated KEGG pathways, as well as the HIF-1α signaling pathway that is known to mediate the host inflammatory response to bacteria (**Figure 2C**). Among the individual upregulated genes (**Table 1**) were the inflammatory mediator *Cxcl5* and its cognate receptor *Cxcr2*, the antimicrobial peptide *Ptx3*, the innate immune cell activating lectin *Clec4e* (also known as Mincle), and *Trem1*, which is expressed on myeloid cells and stimulates release of inflammatory cytokines in response to pathogens (Tessarz and Cerwenka, 2008). Given the gene expression signature of inflammation, we examined whether *Gardnerella*-exposed bladders collected at the same time point used for RNA-seq analysis displayed robust neutrophil migration into the urothelium like what has been seen during bladder infection with established uropathogens (Mulvey et al., 2000; Mulvey et al., 2001). However, we did not observe robust neutrophil infiltration into the urothelium in any of the *Gardnerella*-exposed bladders (**Supplementary Figure S1**), suggesting that either more time or additional exposures may be required to effect changes at the level of neutrophil recruitment, or that a different cellular response occurs. Future time-course

TABLE 1 | Genes differentially expressed in the bladder after two *Gardnerella* exposures.

Gene name	Description	logFC	*p*-value	FDR
Cxcl5	Chemokine (C-X-C motif) ligand 5 [Source : MGI Symbol;Acc : MGI:1096868]	8.11756	3.07E–07	4.98E–04
Trem1	Triggering receptor expressed on myeloid cells 1 [Source : MGI Symbol;Acc : MGI:1930005]	6.00981	1.85E–05	1.09E–02
Syngr4	Synaptogyrin 4 [Source : MGI Symbol;Acc : MGI:1928903]	5.43623	3.22E–05	1.54E–02
Clec4e	C-type lectin domain family 4, member e [Source: MGI Symbol;Acc : MGI:1861232]	5.23586	3.63E–07	5.64E–04
Krt6a	Keratin 6A [Source : MGI Symbol;Acc : MGI:1100845]	4.81622	2.32E–06	2.51E–03
1810065E05Rik	RIKEN cDNA 1810065E05 gene [Source : MGI Symbol;Acc : MGI:1917114]	4.72987	2.13E–05	1.19E–02
Ptpn5	Protein tyrosine phosphatase, non-receptor type 5 [Source : MGI Symbol;Acc : MGI:97807]	4.5585	1.09E–04	3.77E–02
Anxa10	Annexin A10 [Source : MGI Symbol;Acc : MGI:1347090]	4.14225	6.77E–08	1.42E–04
Bdkrb1	Bradykinin receptor, beta 1 [Source : MGI Symbol;Acc : MGI:88144]	4.03099	1.47E–07	2.76E–04
Chrnb4	Cholinergic receptor, nicotinic, beta polypeptide 4 [Source : MGI Symbol;Acc : MGI:87892]	3.95127	1.23E–04	4.11E–02
Fmo4	Flavin containing monooxygenase 4 [Source : MGI Symbol;Acc : MGI:2429497]	3.42047	4.32E–05	1.88E–02
Sprr2g	Small proline-rich protein 2G [Source : MGI Symbol;Acc : MGI:1330348]	3.40759	7.56E–07	1.04E–03
Fosl1	Fos-like antigen 1 [Source : MGI Symbol;Acc : MGI:107179]	3.27974	7.83E–07	1.04E–03
Vat1l	Vesicle amine transport protein 1 homolog-like (*T. californica*) [Source : MGI Symbol;Acc : MGI:2142534]	3.13659	1.53E–04	4.75E–02
Tff1	trefoil factor 1 [Source : MGI Symbol;Acc : MGI:88135]	3.1289	2.34E–05	1.25E–02
Ptx3	Pentraxin-related gene [Source : MGI Symbol;Acc : MGI:104641]	3.07996	2.16E–05	1.19E–02
Cml5	Camello-like 5 [Source : MGI Symbol;Acc : MGI:1916299]	3.06793	3.25E–06	3.23E–03
Cxcr2	Chemokine (C-X-C motif) receptor 2 [Source : MGI Symbol;Acc : MGI:105303]	3.02361	2.04E–06	2.35E–03
Pinlyp	Phospholipase A2 inhibitor and LY6/PLAUR domain containing [Source : MGI Symbol;Acc : MGI:3615324]	3.00226	5.61E–05	2.33E–02
Fam3b	Family with sequence similarity 3, member B [Source : MGI Symbol;Acc : MGI:1270150]	2.97956	1.23E–06	1.56E–03
Mefv	Mediterranean fever [Source : MGI Symbol;Acc : MGI:1859396]	2.97595	3.87E–05	1.75E–02
Gjb4	Gap junction protein, beta 4 [Source : MGI Symbol;Acc : MGI:95722]	2.93731	4.19E–05	1.85E–02
Csta1	Cystatin A1 [Source : MGI Symbol;Acc : MGI:3524930]	2.91392	6.98E–09	2.08E–05
Tnfaip6	Tumor necrosis factor alpha induced protein 6 [Source : MGI Symbol;Acc : MGI:1195266]	2.85446	2.18E–07	3.81E–04
Qrfpr	Pyroglutamylated RFamide peptide receptor [Source : MGI Symbol;Acc : MGI:2677633]	2.84716	1.08E–05	6.92E–03
Gm10309	Predicted gene 10309 [Source : MGI Symbol;Acc : MGI:3641941]	2.82107	1.25E–04	4.12E–02
Rnf183	Ring finger protein 183 [Source : MGI Symbol;Acc : MGI:1923322]	2.76461	8.21E–06	5.98E–03
Krt13	Keratin 13 [Source : MGI Symbol;Acc : MGI:101925]	2.64816	6.48E–05	2.63E–02
Serpina3m	Serine (or cysteine) peptidase inhibitor, clade A, member 3M [Source : MGI Symbol;Acc : MGI:98378]	2.53427	3.16E–06	3.23E–03
Mmp10	Matrix metallopeptidase 10 [Source : MGI Symbol;Acc : MGI:97007]	2.5287	1.30E–04	4.21E–02
Ch25h	Cholesterol 25-hydroxylase [Source : MGI Symbol;Acc : MGI:1333869]	2.49894	1.77E–06	2.15E–03
Socs3	Suppressor of cytokine signaling 3 [Source : MGI Symbol;Acc : MGI:1201791]	2.46996	1.02E–04	3.57E–02
Usp2	Ubiquitin specific peptidase 2 [Source : MGI Symbol;Acc : MGI:1858178]	2.41504	4.93E–10	2.94E–06
Timp1	Tissue inhibitor of metalloproteinase 1 [Source : MGI Symbol;Acc : MGI:98752]	2.22733	5.90E–06	4.79E–03
Lonrf3	LON peptidase N-terminal domain and ring finger 3 [Source : MGI Symbol;Acc : MGI:1921615]	2.20086	1.56E–05	9.61E–03
Nts	neurotensin [Source : MGI Symbol;Acc : MGI:1328351]	2.10926	1.30E–04	4.21E–02
Nr4a2	Nuclear receptor subfamily 4, group A, member 2 [Source : MGI Symbol;Acc : MGI:1352456]	2.10351	3.39E–08	8.08E–05
Errfi1	ERBB receptor feedback inhibitor 1 [Source : MGI Symbol;Acc : MGI:1921405]	2.05576	3.84E–09	1.25E–05
Nr1d1	Nuclear receptor subfamily 1, group D, member 1 [Source : MGI Symbol;Acc : MGI:2444210]	–2.14917	1.41E–13	2.52E–09
2310015D24Rik	RIKEN cDNA 2310015D24 gene [Source : MGI Symbol;Acc : MGI:1917350]	–2.18627	9.74E–05	3.50E–02
Egr1	early growth response 1 [Source : MGI Symbol;Acc : MGI:95295]	–2.28215	2.16E–09	7.72E–06
Snora31	Small nucleolar RNA, H/ACA box 31 [Source : MGI Symbol;Acc : MGI:3819500]	–2.31741	6.27E–06	4.98E–03
Gm15883	Predicted gene 15883 [Source : MGI Symbol;Acc : MGI:3801875]	–2.73333	1.61E–04	4.95E–02
Gm12426	Predicted gene 12426 [Source : MGI Symbol;Acc : MGI:3650989]	–3.28567	9.06E–05	3.41E–02
Fos	FBJ osteosarcoma oncogene [Source : MGI Symbol;Acc : MGI:95574]	–3.8295	2.01E–17	7.18E–13
1200007C13Rik	RIKEN cDNA 1200007C13 gene [Source : MGI Symbol;Acc : MGI:1921369]	–3.85771	8.66E–05	3.37E–02
Gm26887	Predicted gene, 26887 [Source : MGI Symbol;Acc : MGI:5477381]	–4.04586	1.78E–05	1.06E–02
Gm5828	Predicted gene 5828 [Source : MGI Symbol;Acc : MGI:3644176]	–4.35603	8.69E–05	3.37E–02
Igkv12-46	Immunoglobulin kappa variable 12-46 [Source : MGI Symbol;Acc : MGI:4439773]	–7.10718	3.20E–06	3.23E–03

experiments and more in-depth histological analysis by a pathologist and assessment of specific immune cell populations and activation states in the bladder by flow cytometry are needed to distinguish these possibilities.

Upregulated Genes and Pathways Reflect *Gardnerella*-Induced Urothelial Exfoliation

Several significantly upregulated gene sets were related to urothelial integrity and turnover. 'DNA replication' was the top hit in both the KEGG and GO biological process lists (**Figure 2**). Additional terms indicating cell proliferation in the *Gardnerella*-exposed bladders were those related to cell cycle, ribosome biogenesis, translation, cell activation, and nuclear division (**Supplementary Tables S2–S4**). The "neuroactive ligand–receptor interaction," "cytokine–cytokine receptor interaction," and "retinol metabolism" (Lu et al., 2021) KEGG pathways have been linked to bladder cancer (Zhang et al., 2021). Several of the individual genes upregulated by *Gardnerella* exposures are involved in epithelial to mesenchymal transition (*Cxcl5*, *Cxcr2*, *Tff1*) (Lee et al., 2021) or known to be elevated during squamous metaplasia or bladder cancer (*Anxa10*, *Fosl1*, *Krt6a*, *Mmp10*) (Cao et al., 2010; Somji et al., 2011; Gatta et al., 2019; Kudelski et al., 2021; Wu et al., 2021). These RNA-seq data are consistent with our previous findings, reproduced here, that

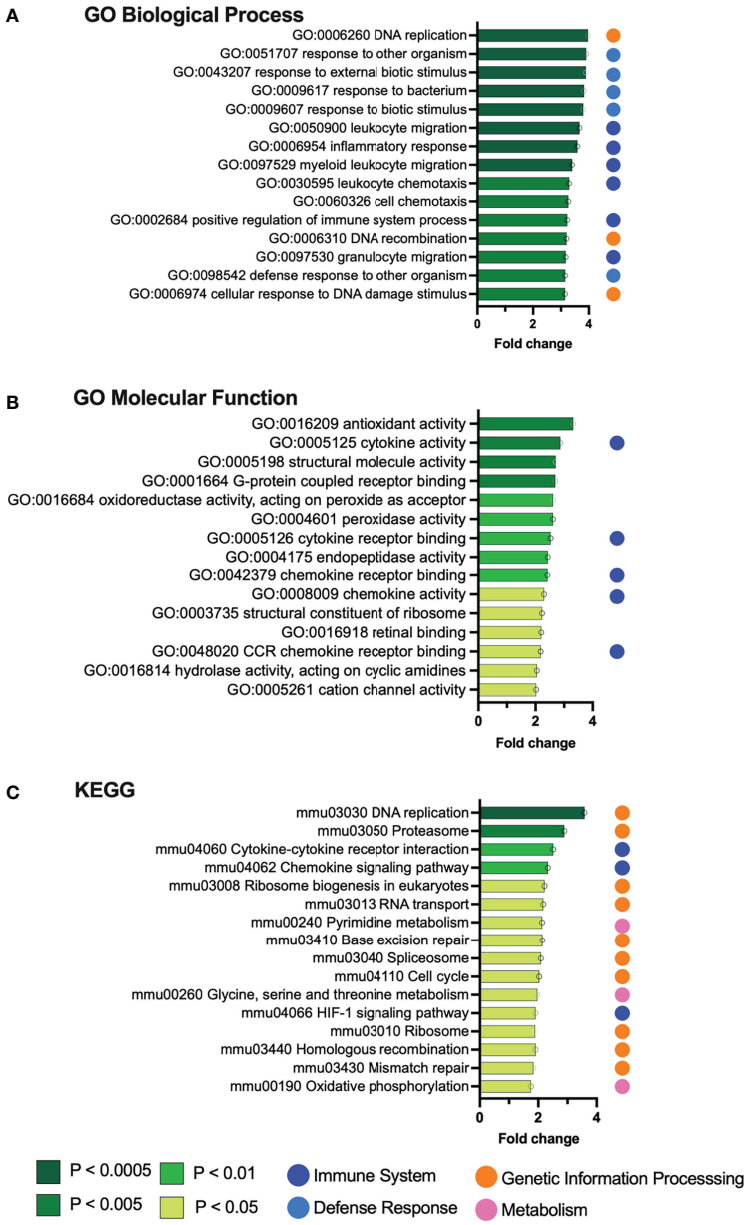

FIGURE 2 | Gene set enrichment analysis identifies pathways upregulated by *Gardnerella* exposures. Graphs depict the top 15 gene sets from the Gene Ontology **(A, B)** and KEGG **(C)** databases that were significantly enriched in the bladders of mice exposed to *Gardnerella* relative to PBS controls.

two *Gardnerella* exposures trigger membrane blebbing and exfoliation of superficial umbrella cells lining the bladder lumen (**Figure 3A**) (Gilbert et al., 2017). To further corroborate the RNA-seq signature of urothelial turnover, additional bladders were examined for markers of urothelial differentiation and proliferation. Compared to bladders exposed only to PBS, three out of four bladders exposed to *Gardnerella* displayed a noticeable increase in urothelial keratin 6 (Krt6), a marker of squamous differentiation, which is consistent with the increased *Krt6* transcript detected by RNA-seq (**Supplementary Figure S2**). Another marker of urothelial differentiation is cytokeratin 20: its appearance on the apical membrane marks the last event in differentiation of superficial umbrella cells (Veranic et al., 2004). Bladders from mice exposed only to PBS had cytokeratin 20 (CK20) staining that was contiguous across the length of the urothelial-luminal interface, indicating an intact and fully differentiated urothelium (**Figure 3B** (I). In contrast, the urothelium from mice exposed to *Gardnerella* had regions lacking CK20 staining (**Figure 3B** (II and III). We enumerated CK20-positive superficial cells lining the urothelial surface. We

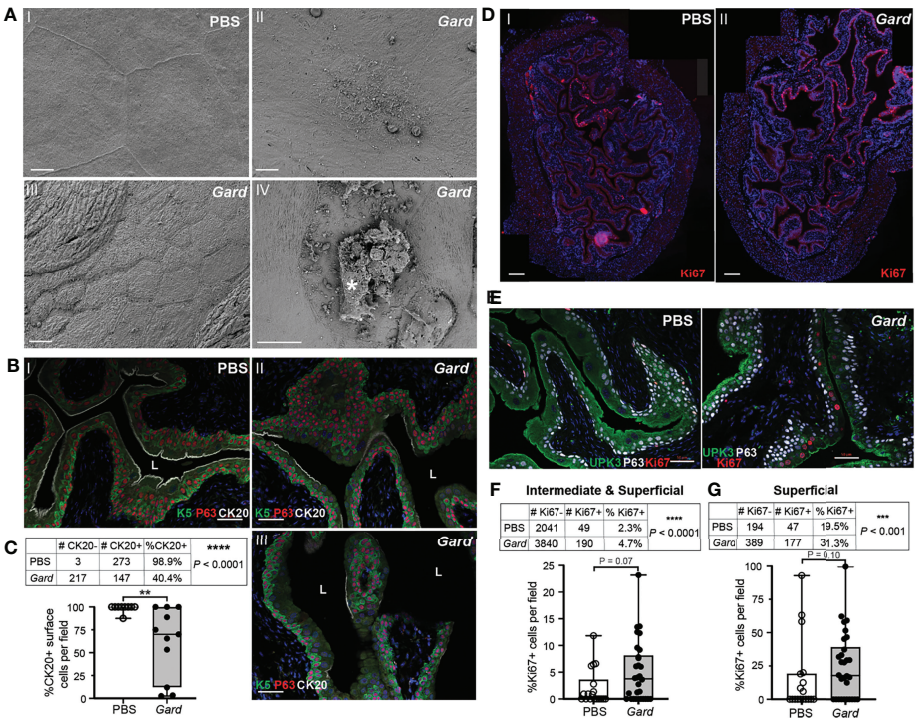

FIGURE 3 | *Gardnerella* exposures result in urothelial exfoliation and proliferation. **(A)** Scanning electron micrographs of bladders collected 12 h after two exposures (the model used for RNA-seq, see **Figure 1** schematic) to PBS (panel I) or *Gardnerella* (II–IV). (I) Intact superficial umbrella cells; (II) umbrella cell with membrane blebbing; (III) region of exfoliation with smaller underlying intermediate cells visible at the luminal interface; (IV) exfoliating umbrella cell marked by an asterisk. Scale bars = 10 μm. **(B)** Immunofluorescence microscopy of urothelial differentiation markers in bladder sections from mice exposed to PBS (I) or *Gardnerella* (II and III). Keratin 5 (K5) in green labels, basal cells; P63 in red labels, basal and intermediate cells; and cytokeratin 20 (CK20) in white labels, the apical surface of fully differentiated umbrella cells. Scale bars = 50 μm. **(C)** Counts of CK20-positive (+) and CK20-negative (–) cells on the urothelial surface, adjacent to the lumen. $^{****}p < 0.001$, Fisher's exact test. The graph was generated by plotting the %CK20 positivity, with each dot representing an individual microscopy image of the ×20 field of view. $^{**}p < 0.01$, Mann–Whitney *U* test. Box plot denotes the 25th and 75th percentiles with a line at the median and whiskers from min to max. **(D)** Panoramic assembly of bladder sections stained for Ki67 in red. Scale bars = 200 μm. **(E)** Representative images of immunofluorescence microscopy used for Ki67+ enumeration. **(F, G)** Tables show the counts of Ki67-positive (+) and Ki67-negative (–) cells of superficial and intermediate cells combined **(F)** or only superficial cells **(G)** in each experimental group. $^{***}p < 0.001$, $^{****}p < 0.0001$, Fisher's exact test. Graphs were generated by plotting the %Ki67 positivity, with each dot representing an individual microscopy image as in **(B)**. Box plot denotes the 25th and 75th percentiles with a line at the median and whiskers from min to max.

also counted the total number of superficial cells (those at the luminal interface) in order to determine the percentage that were CK20 positive. These data confirmed a significant decrease in CK20 staining in mice exposed to *Gardnerella* compared to PBS controls (**Figure 3C**). The increased Krt6 and decreased CK20 staining pattern observed on *Gardnerella*-exposed bladders suggested that exfoliation had occurred in these areas and the urothelium had not completely healed. Finally, we examined cell proliferation *via* Ki67 staining. Naive, unperturbed adult mouse bladders have a very slow urothelial turnover and thus contain few to no Ki67-positive cells. Exfoliation of superficial urothelial cells is known to trigger proliferation and differentiation of underlying cells to restore the urothelium. In our model, Ki67-positive cells were present in *Gardnerella*-exposed bladders (**Figure 3D** (II), but they were also seen in bladders exposed to PBS, reflecting the fact that the transurethral inoculation procedure itself perturbs the bladder tissue (**Figure 3D** (I). To determine whether there was an overall increase in proliferation in *Gardnerella*-exposed mice compared to PBS controls, we enumerated Ki67-positive cells in the transitional urothelium (**Figures 3E–G**). We also counted the total number of cells in order to determine the percentage that were Ki67 positive. When the transitional urothelium was analyzed (intermediate and superficial cells), the proportion of Ki67-positive cells was significantly higher in mice exposed to *Gardnerella* than in PBS controls (**Figure 3F**). This increase in Ki67 positivity in *Gardnerella*-exposed bladders was apparent even if only superficial cells were analyzed (**Figure 3G**). Our previous data suggested that exfoliating cells are dying *via* apoptosis since *Gardnerella*-exposed bladders exhibited increased cleaved Casp-3 staining and TUNEL-positive urothelial cells (Gilbert et al.). Consistent with this phenotypic data, *Gardnerella* exposures increased 15 GO terms related to apoptosis (**Supplementary Table S2**). These data, together with our previous findings, demonstrate that bladder exposures to *Gardnerella* result in urothelial exfoliation.

Gardnerella Preexposures Promote Acute UPEC Bacteriuria

Urothelial turnover and inflammatory responses in the bladder are important features of UTI caused by established uropathogens such as UPEC (Lacerda Mariano and Ingersoll, 2020). Given our RNA-seq results, we hypothesized that *Gardnerella* exposure would affect the course of an experimental UPEC UTI. To examine this hypothesis, we developed a "preexposure" model in which mice received *Gardnerella* exposures before UPEC inoculation (**Figure 4A**). We exposed mice twice, 12 h apart, to *Gardnerella* as in the RNA-seq experiment. Twelve hours after the second *Gardnerella* or PBS control preexposure, mice were inoculated transurethrally with the UPEC clinical isolate UTI89. The UTI89 strain is widely used in experimental UTI models, including in our prior study that reported that *Gardnerella* exposures induced recurrent UPEC UTI from intraepithelial reservoirs (Gilbert et al., 1986).

The UPEC pathogenic cascade involves urothelial attachment, invasion, and intracellular replication followed by filamentation and egress (Klein and Hultgren, 2020). We hypothesized that the urothelial exfoliation resulting from *Gardnerella* exposure could disrupt or alter the interaction of UPEC with the urothelium. First, we directly examined UPEC-bladder interactions in our preexposure model using scanning electron microscopy (SEM). Consistent with previous reports in naive mice, UPEC-infected mice preexposed to PBS harbored abundant UPEC adhering to, and filamenting out of, superficial urothelial cells as well as exfoliating cells full of intracellular bacteria (**Figure 4B**, top). Similar features were also seen on the superficial cells of bladders preexposed to *Gardnerella* (**Figure 4B**, bottom), suggesting that the urothelium was still able to harbor intracellular UPEC. One noticeable difference was that some of the superficial cells in bladders preexposed to *Gardnerella* had small collections of adherent bacillary UPEC in addition to the filamentous bacteria more typically observed in

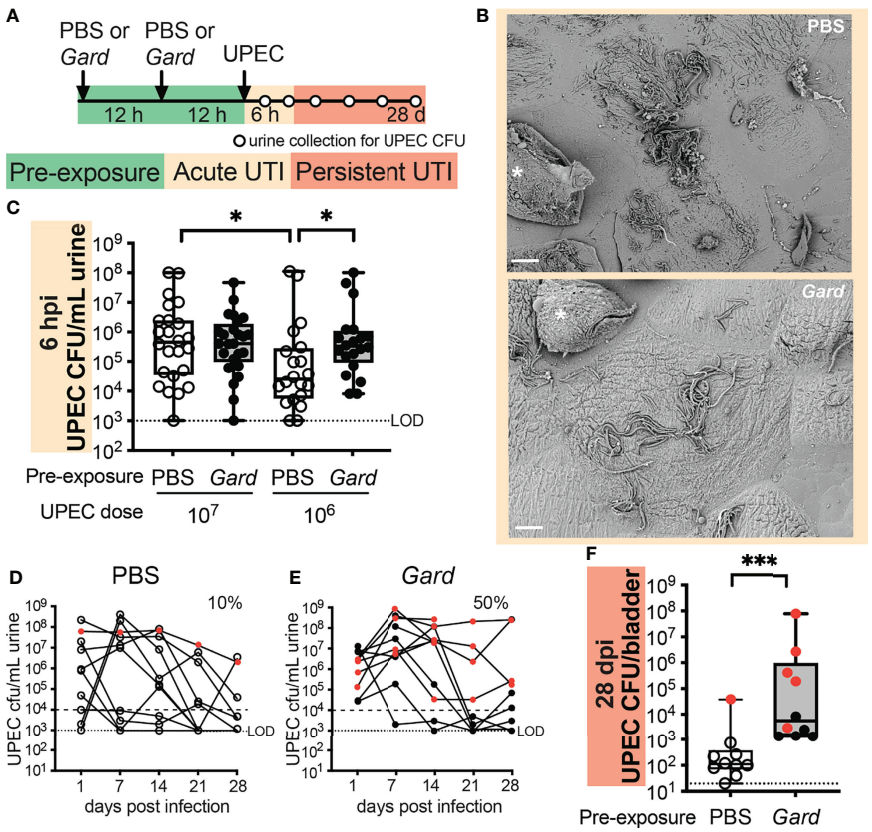

FIGURE 4 | Preexposure to *Gardnerella* increases acute UPEC bacteriuria and persistent infection. **(A)** Schematic of the mouse experiment time course. Dots on the timeline indicate when urine was collected for CFU enumeration. Data are from five independent mouse experiments. **(B)** SEM images of bladders collected 6 h after UPEC infection from mice preexposed to PBS (top) or *Gardnerella* (bottom). Asterisks mark exfoliating umbrella cells. Scale bars = 20 μm. Refer to **Supplementary Figure S3** for additional bladder SEM images. **(C)** Acute UPEC titers in urine collected 6 hpi, with each dot representing an individual mouse (10^7 n = 20 per group; 10^6 PBS n = 24, *Gardnerella* n = 25). *$p < 0.05$, Mann–Whitney U test. Box plot denotes the 25th and 75th percentiles with a line at the median and whiskers from min to max. **(D, E)** Time course of UPEC bacteriuria after a 10^7 CFU inoculation. Red symbols indicate mice that had UPEC bacteriuria >10^4 CFU/ml in each weekly urine sample. **(F)** UPEC titers in bladder homogenates collected 28 dpi. Each dot represents an individual mouse (n = 10 per group). Red symbols indicate mice that had UPEC bacteriuria >10^4 CFU/ml in each weekly urine sample. LOD, limit of detection. ***$p < 0.001$, Mann–Whitney U test. Box plot denotes the 25th and 75th percentiles with a line at the median and whiskers from min to max.

control bladders (**Supplementary Figure S3**, arrowheads). Ultimately, the SEM analysis demonstrated that preexposure to *Gardnerella* did not entirely disrupt the UPEC invasion and egress cycle.

Next, we took a more quantitative approach by examining UPEC titers in urine in our preexposure model. With an inoculum of 10^7 colony-forming units (CFUs) of UPEC, which is frequently used in mouse UTI models (Conover et al., 2015; Gilbert et al.), UPEC urine titers were indistinguishable between PBS preexposed and *Gardnerella* preexposed mice at 6 and 24 hpi (**Figure 4C**; **Supplementary Figure S4**). We reasoned that the effects of *Gardnerella* might be masked in the context of the high titer UPEC inoculum, especially at early time points. Therefore, we also examined a lower dose of 10^6 CFUs of UPEC. Strikingly, UPEC bacteriuria was significantly higher at 6 hpi in mice preexposed to *Gardnerella* than in PBS preexposed controls (**Figure 4C**). These data suggest that alterations in the bladder niche caused by preexposures to *Gardnerella* promote UPEC bacteriuria.

Gardnerella Exposures Promote UPEC Persistence in the Bladder

Since we observed an effect of *Gardnerella* preexposures on acute bacteriuria, we examined whether this would extend to later time points and whether the effect would be observable in bladder tissue in addition to urine. Wild-type C57BL/6 female mice are generally not susceptible to persistent (chronic) bacteriuria following a single UPEC inoculation (Schwartz et al., 2015). Consistent with prior reports, most of the mice inoculated with 10^6 CFUs of UPEC cleared bacteriuria by 24 hpi, with no apparent difference between preexposure groups (**Supplementary Figure S4**). With an inoculum of 10^7 CFUs of UPEC, only 10% of mice in the PBS control preexposure group had UPEC bacteriuria that persisted at $>10^4$ CFU/ml out to 4 weeks postinfection (wpi) (**Figure 4D**), compared with 50% in mice preexposed to *Gardnerella* (**Figure 4E**). Previous studies have shown that mice that develop persistent bacteriuria have higher levels of the proinflammatory cytokines IL-5, IL-6, KC, and G-CSF in serum at 24 hpi compared to those that ultimately clear the infection (Hannan et al., 2010; Schwartz et al., 2015). We hypothesized that *Gardnerella* exposure alone may result in a similar cytokine signature, and if this were to occur prior to UPEC introduction into the bladder, it could prime the host for the development of persistent UPEC infection. To test this hypothesis, we measured cytokine levels in bladders that were exposed twice to *Gardnerella* and were collected at the time point that they would have received UPEC in our preexposure model. However, inconsistent with our hypothesis, there was no difference in serum levels of IL-5, IL-6, KC, or G-CSF (**Supplementary Figure S5**) or in any of the other 23 cytokines/chemokines measured (data not shown) in mice preexposed to *Gardnerella* versus to PBS.

Bladder titers of UPEC at 4 wpi were significantly higher in mice exposed to *Gardnerella* compared to PBS controls ($p = 0.0003$; **Figure 4F**). This difference remained significant even when the bladders from mice without persistent UPEC bacteriuria were analyzed separately (**Supplementary Figure S6**). This is notable because UPEC that is detectable in bladder tissues in the absence of bacteriuria has previously been shown to reside in quiescent intracellular reservoirs (Mulvey et al., 2001), suggesting that preexposure to *Gardnerella* may promote the formation of more, or larger, reservoirs. Future studies using methods that distinguish intracellular from luminal UPEC are needed to distinguish these possibilities. Taken together, these initial studies demonstrate that preexposure to *Gardnerella* promotes both acute and persistent UPEC infection in the bladder.

DISCUSSION

Gardnerella is a common, and often dominant, member of the vaginal microbiome, especially in the context of dysbiosis (Morrill et al., 2020). Likewise, *Gardnerella* has recently been recognized as a frequent, dominant member of the urobiome, including in studies that used collection methods to limit contamination by periurethral or vaginal organisms (Birch et al., 1981; McDowall et al., 1981; McDonald et al., 1982; Fairley and Birch, 1983; Gilbert et al., 1986). Despite *Gardnerella* being frequently isolated from urine, relatively little is known regarding the biological effects of *Gardnerella* on bladder tissue. To address this lack of knowledge, we recently developed a model of *Gardnerella* bladder exposure in female mice (O'Brien et al., 2020). Here, we further characterized our model using whole bladder RNA-seq to determine the bladder transcriptomic response to *Gardnerella*. The RNA-seq data, along with additional microscopy validation studies, echo our previous report of *Gardnerella*-induced urothelial apoptosis and exfoliation (Gilbert et al., 2017). The RNA-seq results further revealed that *Gardnerella* activates host transcriptional pathways related to mucosal inflammation and immunity. Taken together, these data provide evidence that bacterial species such as *Gardnerella*, that have been frequently identified in urinary microbiome studies but have remained understudied, have observable biological effects on the bladder tissue in a relevant *in vivo* model.

To further explore the potential clinical relevance of *Gardnerella* bladder exposures, here, we focused on the effect of *Gardnerella* on UTIs caused by UPEC. We chose to focus first on UPEC UTI for the following reasons: First, women with BV, who thus have high levels of *Gardnerella*, are at increased risk of UTI (Hooton et al., 1989; Sumati and Saritha, 2009; Hillebrand et al., 2022). Second, sexual activity, which likely results in bladder exposure to urogenital bacteria like *Gardnerella*, is one of the strongest risk factors for UTI (Nicolle et al., 1982; Foxman, 2014). Of particular relevance to these first two points, one study found that the strongest correlation between the vaginal and urinary microbiome occurred in women with BV (Gottschick et al.). Third, modulation of the urobiome has been proposed as an antibiotic-sparing alternative therapeutic approach to treat or prevent UTIs (Jung and Brubaker, 2019; Cole et al., 2021; Jones et al., 2021; Garofalo et al., 2022), but the direct impact of urobiome members on UTI outcomes have not been examined *in vivo*. Fourth, the most notable effects of *Gardnerella* in our RNA-

seq dataset were urothelial integrity and inflammation, which are known to be key host determinants of UPEC UTI. In summary, the convergence of data from clinical studies and our *Gardnerella* bladder exposure model led us to extend our model to examine the effects of *Gardnerella* exposure on outcomes of UTIs caused by UPEC. Data from this model provide evidence that preexposures to *Gardnerella* can enhance UPEC acute and persistent UTIs. *Gardnerella* preexposures enhanced UPEC bacteriuria, at an early time point, in a manner dependent on UPEC dose. The effect of *Gardnerella* on acute UPEC bacteriuria was only evident when mice were given a relatively lower UPEC inoculum. This observation suggests that *Gardnerella* bladder exposures could lower the threshold dose required for UPEC to establish UTI in women. Additionally, preexposure to *Gardnerella* rendered mice more susceptible to persistent UPEC bacteriuria and increased UPEC burden in bladder tissue. The increase in bladder UPEC was detected even in mice without persistent UPEC bacteriuria. The presence of UPEC in bladder tissue in the absence of bacteriuria has previously been attributed to stable UPEC reservoirs within urothelial cells that can later emerge to cause a recurrent UTI (Mulvey et al.). The data presented here warrant future studies using established assays (e.g., immunofluorescence microscopy, gentamicin protection) to examine UPEC intracellular niche distribution in the context of *Gardnerella* exposure. Taken together, these data demonstrate that bladder exposures to *Gardnerella* enhance UPEC UTI in a relevant *in vivo* model and provide further biological explanation for the association between BV and UTI observed in women.

While it is evident from the data presented here that *Gardnerella* exposures enhance UPEC UTI, the molecular mechanisms driving this effect remain to be determined. Despite *Gardnerella* activating transcription of inflammatory pathways, we observed no evidence of tissue inflammation. However, it remains possible that distinct immune cell populations are responding to *Gardnerella* in ways that require more focused assays to detect. So far, the data point to the effect of *Gardnerella* on the urothelium as the most likely source of influence on UPEC UTI. Future experiments could test this idea by attempting to block exfoliation using cell-death pathway inhibitors. Prior studies have pointed to host responses happening during the acute stages of UPEC infection as drivers of chronic outcomes (Hannan et al., 2010; Schwartz et al., 2015). We expect that a similar situation is occurring in our preexposure model, meaning that whatever is promoting UPEC persistence in *Gardnerella*-exposed mice occurs during the early stages of UPEC infection. We do not expect that *Gardnerella* directly impacts the bladder tissue 1 month after exposure because *Gardnerella* is cleared from the mouse urinary tract by 12 h (Gilbert et al.).

A limitation of our study was that we only examined a single time point. Also, since we examined bladders 12 h after two exposures to *Gardnerella*, we cannot distinguish whether the differences required two exposures or if they would have occurred 24 h following one exposure. Since whole bladders were analyzed, we cannot attribute transcriptional changes to specific regions or cell types of the bladder, and RNA-seq analysis will not uncover epigenetic changes that have occurred. Another limitation was that we only examined one strain of *Gardnerella* (JCP8151B) that is a vaginal isolate and which may become reclassified as a species other than *vaginalis* (but was regarded as *G. vaginalis* when we performed our study). Studies examining associations between *Gardnerella* in the urobiome and various conditions associated with lower urinary tract symptoms, such as urgency urinary incontinence and overactive bladder, have yielded mixed results. However, many of these studies only examined the urobiome at the genus level and did not distinguish between *Gardnerella* species or subgroups. It is possible that the inconsistency in associations with lower urinary tract symptoms reflects that different *Gardnerella* species have greater or less capacity to influence the bladder. It has been noted that *G. piotii* has not yet been isolated from urine, while most other species and subspecies of *Gardnerella* have been isolated from both niches (Putonti et al., 2021). However, this could be attributed to differences in the culture techniques that were used in studies isolating *Gardnerella* from the urine compared to those that isolated *Gardnerella* from the vagina. Whether or not different *Gardnerella* species or subgroups display different colonization kinetics or pathologic features in the bladder can be directly tested in mice using our *Gardnerella* exposure model.

In addition to the connection to UTI, the genes and biological pathways affected by *Gardnerella* in our mouse model are related to bladder function and intersect with a wide range of other urological conditions, including but not limited to bladder cancer, urinary incontinence, bladder pain syndrome and interstitial cystitis. For example, cholinergic and bradykinin receptors and the neuropeptide neruotensin (Nts) mediate bladder muscle cell contraction (Dong et al., 2015; Dalghi et al., 2020; Borsodi et al., 2021). Type 1 bradykinin receptors like Bdkrb1 are generally not expressed in healthy tissue but are induced by inflammatory mediators and injury (Marceau et al., 1997), which is consistent with the increase in expression after *Gardnerella* exposure. The nicotinic cholinergic receptor that was induced by *Gardnerella*, Chrnb4, is expressed by bladder afferent neurons and is necessary for strips of bladder tissue to contract in response to nicotine stimulation *ex vivo* (Xu et al., 1999). The Human Phenotype Ontology database reports that the orphan nuclear receptor Nr4a2 is associated with bladder function and urinary urgency. Dysregulation of the "neuroactive ligand–receptor interaction" and "phototransduction" KEGG pathways further suggests an influence of *Gardnerella* exposures on the bladder–brain axis. The observation that *Gardnerella* exposure induced genes related to bladder sensation and urination is noteworthy because some studies in women have detected an association between the presence of *Gardnerella* in urine and urgency urinary incontinence. Future studies could expand the exposure model to investigate the effect of *Gardnerella* on urination frequency and other measures of bladder function.

In summary, here, we demonstrate that *Gardnerella* directly impacts the bladder, activating transcriptional inflammatory

responses and causing urothelial exfoliation and turnover. We present further evidence of *Gardnerella* as a "covert pathogen" in the bladder (Gilbert and Lewis, 2019), affecting outcomes of UPEC UTI at time points long after *Gardnerella* has been cleared from the bladder. These findings have important implications for how we think about the potential influence of urobiome bacteria on disease outcomes in the bladder.

MATERIALS AND METHODS

Ethics Statement

Mouse experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The Institutional Animal Care and Use Committee (IACUC) of Washington University School of Medicine approved all procedures in advance (Protocol Numbers: 20170081 and 20-0031).

Bacterial Strains and Growth Conditions

Gardnerella strain JCP8151B (Lewis et al., 2013) was grown anaerobically at 37°C in static liquid culture in NYCIII medium for 16 h or on NYCIII agar plates with 1 mg/ml streptomycin. Uropathogenic *E. coli* strain UTI89, harboring a kanamycin resistance cassette (Wright et al., 2005), was grown aerobically at 37°C in static liquid culture in Lysogeny Broth (LB) medium for 18 h and subcultured 1:1,000 in fresh LB for 18 h or on LB agar plates with 25 mg/ml kanamycin. Mouse inocula were prepared as previously described (O'Brien et al., 2020).

Mice

Six- to seven-week-old female C57BL/6 mice were obtained from Charles River (Fredericks facility). Mice were given a regular chow diet in a specific pathogen-free facility with a 12-h light/12-h dark cycle at Washington University School of Medicine. Mice were allowed to acclimate to the facility after transport for 1 week prior to experiments.

Mouse Urinary Tract Inoculation Experiments for RNA-seq

Experiments were performed essentially as described previously (O'Brien et al., 2020). Briefly, mice were anesthetized with isoflurane and then inoculated transurethrally with 50 µl prepared inoculum of 1×10^8 CFU *Gardnerella* strain JCP8151B (5 mice) or PBS (4 mice). Twelve hours later, mice received a second transurethral inoculation of *Gardnerella* or PBS. Twelve hours later, all mice were humanely sacrificed by cervical dislocation under isoflurane anesthesia, and bladders were aseptically harvested and flash frozen in liquid nitrogen for future RNA isolation.

Library Preparation and Sequencing

Bladders were homogenized and RNA was extracted using the RNeasy Plus Mini kit (Qiagen). Libraries were prepared from each bladder individually with 10 ng of total RNA, and RNA integrity was determined using an Agilent Bioanalyzer, with a Bioanalyzer RIN score >8.0 obtained for all samples. ds-cDNA was prepared using the SMARTer Ultra Low RNA Kit for Illumina Sequencing (Takara-Clontech) per the manufacturer's protocol. cDNA was fragmented using a Covaris E220 sonicator using peak incident power of 18, duty factor 20%, cycles/burst 50, time 120 s to yield an average size of 200 base pairs (bp). cDNA was then blunt ended, had an A base added to the 3′ ends, and then had Illumina sequencing adapters ligated to the ends. Ligated fragments were then amplified for 12 cycles using primers incorporating unique index tags. Fragments were multiplexed with 5–6 samples per lane and were sequenced on an Illumina HiSeq 2500 using single-end 50 bp reads to target 30 M reads per sample.

RNA-seq Data Acquisition, Quality Control, and Processing

RNA-seq reads from the nine individual libraries were demultiplexed using a custom demultiplexing script written in Python and then aligned to the Ensembl GRCm38.76 (*Mus musculus*) assembly with STAR version 2.0.4b. Subread: featureCount version 1.4.5 was used to derive gene counts from the number of uniquely aligned unambiguous reads. Sailfish version 0.6.3 was used to produce transcript counts. RSeQC version 2.3 was used to assess sequencing performance for total number of aligned reads, total number of uniquely aligned reads, genes and transcripts detected, ribosomal fraction, known junction saturation, and read distribution over known gene models. All gene-level and transcript counts were then imported into the R/Bioconductor package EdgeR and TMM-normalized to adjust for differences in library size. Genes or transcripts not expressed in any sample were excluded from further analysis. Spearman correlation matrix and multidimensional scaling plots were used to assess the performance of the samples. Generalized linear models with robust dispersion estimates were created to test for gene/transcript level differential expression. The fits of the trended and tagwise dispersion estimates were then plotted to confirm proper fit of the observed mean to variance relationship where the tagwise dispersions are equivalent to the biological coefficients of variation of each gene. Differentially expressed genes and transcripts (comparing PBS vs. *Gardnerella*) were then filtered for FDR-adjusted *p*-values less than or equal to 0.05. Global perturbations in known GO terms and KEGG pathways were detected for each EdgeR contrast using the R/Bioconductor package GAGE to test for changes in expression of the reported $\log_2$ fold-changes reported by edgeR in each term versus the background $\log_2$ fold-changes of all genes found outside the respective term.

Deparaffinization and Antigen Retrieval

Additional bladders were collected and fixed overnight in 4% paraformaldehyde at 4°C with gentle shaking and then transferred to 70% ethanol. Bladders were embedded in paraffin and sagittal sections were prepared and mounted on glass slides. Slides were placed onto glass holding trays and then placed into fresh Histo-Clear® Histological Clearing Agent,

National Diagnostics, for 10 min two times. The trays were drained, then moved to 100% ethyl alcohol for 10 min two times, and then to 95% ethyl alcohol two times for 10 min. Finally, glass trays holding the slides were placed under running water for 10 min. During the deparaffinization, fresh pH 9 and 6 buffered antigen retrieval solutions were made and brought to a boil in 50 ml BD conical tubes in a glass beaker filled with water in a steamer. After washing, glass slides were placed into the appropriate buffer around 90°C–100°C without allowing the slides to dry out and boiled for 15 and 30 min in for pH 9 and pH 6 buffer, respectively. Slides were then cooled and allowed to cool to 60°C and were washed in 0.5% Triton X-100 in phosphate-buffered saline (PBS) at room temperature.

Antibody and Histology Staining

For histological analysis, slides were stained with hematoxylin and eosin (H&E) according to standard protocol. For immunostaining, glass slides were removed from wash buffer one at a time, placed horizontally into humidified slide boxes, and the hydrophobic boundary was marked around the perimeter with a PAP PEN. About 300 µl of 10% heat-inactivated horse serum (HIHS) and 3% bovine serum albumin (BSA) in 0.5% Triton X-100 PBS were placed onto each slide for blocking. Slides were incubated in a closed, humidified slide box for 1 to 2 h. During blocking, primary antibody cocktails of chicken anti-KRT 5 1:500; goat anti-P63 1:300; mouse anti-cytokeratin 20 1:200 or rabbit anti-Krt6a 1:1,500; rabbit anti-Ki67 1:200; and mouse anti-Upk3 1:50 were prepared in 1% HIHS and 1% BSA in 0.5% Triton X-100 sufficient for around 300 µl per slide. The blocking solution was removed by vacuum and the primary antibody added onto the slide without disturbing hydrophobic perimeter. Slides were incubated overnight in humidified slide boxes at 4°C.

The following day, the primary antibody was removed, and the slides were washed for 10 min in fresh 0.5% Triton X-100 PBS twice. During washes, secondary antibody cocktails were prepared in1% HIHS and 1% BSA in 0.5% Triton X-100 for 300 µl per slide, and two drops of NucBlue® nuclear staining reagent (DAPI) were added per milliliter of antibody cocktail. Slides were removed from the washing buffer and hydrophobic perimeters redrawn then a secondary antibody cocktail was added. Slides were incubated in the dark at room temperature for 30 min to 1 h. After incubation, slides were washed, and previously warmed DAKO glycerol mounting medium was applied before the coverslip. Slides were stored overnight at 4C in slide folders.

Immunofluorescent and Brightfield Imaging and Analysis

Fluorescent images were acquired with a Zeiss Axiovert 200M microscope with Zeiss Apotome as previously described (Tate et al., 2021). Urothelium cell types were distinguished and counted as: basal cells Krt5+P63+, intermediate cells P63+Krt5−, and superficial cells were P63−CK20+. The analysis of CK20 staining used 2 mice per experimental group, with three nonadjacent sections counted from each bladder. Cells at the luminal interface were enumerated as CK20+ or CK20− in 1–2 images from each bladder section (>275 total cells counted per group). The analysis of Ki67 staining used 3 mice per experimental group, with two nonadjacent sections counted from each bladder. Superficial (C20+P63−) and intermediate cells (P63+Krt5−) were enumerated as Ki67+ or Ki67− in 3–5 images from each bladder section (>2,000 total cells counted per group). Bright-field images were collected using a Nikon Eclipse TE200 microscope. Data were analyzed using the Fiji package of ImageJ.

Scanning Electron Microscopy

Bladders were fixed *in situ* with EM fixative (2% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4) as previously described (O'Brien et al., 2020). Samples were postfixed in 1.0% osmium tetroxide, dehydrated in increasing concentrations of ethanol, and then dehydrated at 31.1°C and 1,072 PSI for 16 min in a critical point dryer. Bladders were quadrisected to reveal the urothelial surface and were mounted on carbon tape-coated stubs and sputter-coated with gold/palladium under argon. Bladders were imaged on a Zeiss Crossbeam 540 FIB-SEM.

Mouse Model Examining the Effects of *Gardnerella* "Preexposures" on UPEC UTI

Mice were anesthetized with isoflurane and then inoculated intravesically twice, 12 h apart, with either 50 µl of prepared 1×10^8 *Gardnerella* strain JCP8151B inoculum or PBS as a control, as described above. Twelve hours after the second inoculation, mice were inoculated with 50 µl of prepared inoculum containing either 1×10^7 or 1×10^6 UPEC strain UTI89kanR. To monitor acute UPEC infection, urine was collected at 6 and 24 h after UPEC inoculation, and titers were enumerated by serial dilution and plating on LB + kanamycin selective media. A subset of mice was monitored for UPEC persistence in the urinary tract by enumerating UPEC in urine weekly out to 4 weeks postinfection. At 4 wpi, mice were humanely sacrificed by cervical dislocation under isoflurane anesthesia, and bladders were aseptically harvested. Homogenates were prepared in 1 ml of sterile PBS and plated on selective media. Samples with no colonies were plotted at the limit of detection.

Cytokine and Chemokine Analysis

Cytokine content was measured in mouse serum using the Bio-Plex-Pro Mouse Cytokine 23-Plex, Group I Panel Multiplex Cytokine Bead Kit (Bio-Rad), which quantifies the following 23 cytokines and chemokines: IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, Eotaxin, G-CSF, GM-CSF, IFN-γ, KC, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α. The assay was performed according to manufacturer instructions, except using a tenfold less standard and half the amount of coupled beads and detection antibodies indicated in the protocol.

DATA AVAILABILITY STATEMENT

The data presented in this study are deposited in the GEO repository, accession number GSE203195.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Washington University School of Medicine.

AUTHOR CONTRIBUTIONS

NG and VO performed RNA-seq experiments. NG performed preexposure experiments with UPEC, measured cytokines, and performed SEM. CW and EB performed histology and immunofluorescence microscopy. CW enumerated Ki67+ cells. NG, CM, and AL analyzed the data. NG and VO drafted the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.909799/full#supplementary-material

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Commensal Urinary Lactobacilli Inhibit Major Uropathogens *In Vitro* With Heterogeneity at Species and Strain Level

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The human urinary microbiome is thought to affect the development and progression of urinary tract infections (UTI), particularly recurrent UTIs in aging populations of women. To understand the possible interactions of urinary pathogens with commensal bacteria inhabiting the aging bladder, we conducted an initial functional assessment of a representative set of urinary lactobacilli that dominate this niche in postmenopausal women. We created a repository of urinary bladder bacteria isolated *via* Enhanced Quantitative Urinary Culture (EQUC) from healthy postmenopausal women, as well as those with a culture-proven recurrent UTI (rUTI) diagnosis. This repository contains lactobacilli strains from eight different species. As many other lactobacilli are known to inhibit human pathogens, we hypothesized that some urinary lactobacilli will have similar abilities to inhibit the growth of typical uropathogens and thus, provide a link between the urinary microbiome and the predisposition to the rUTI. Therefore, we screened the urinary lactobacilli in our repository for their ability to inhibit model uropathogens *in vitro*. We observed that many urinary isolates strongly inhibit model strains of gram-negative *Escherichia coli* and *Klebsiella pneumoniae* but demonstrate less inhibition of gram-positive *Enterococcus faecalis*. The observed inhibition affected model strains of uropathogens as well as clinical and multidrug-resistant isolates of those species. Our preliminary analysis of inhibition modes suggests a combination of pH-dependent and cell-dependent inhibition. Overall, inhibition strongly varies among species and strains of urinary lactobacilli. While the strength of the inhibition is not predictive of health outcomes in this limited repository, there is a high level of species and strain diversity that warrants future detailed investigations.

Keywords: urinary microbiome, commensal lactobacilli, urinary lactobacilli, urinary tract infection, uropathogens

INTRODUCTION

Urinary tract infections (UTIs) are among the most abundant bacterial infections worldwide, with over 6 million annual diagnoses in the U.S. alone (Flores-Mireles et al., 2015; Klein and Hultgren, 2020). The majority of UTIs are caused by enteric bacteria that may originate from the gastrointestinal tract, such as *Escherichia coli, Klebsiella pneumoniae*, and *Enterococcus faecalis* (Thomas-White et al., 2018). The rise of antibiotic-resistant uropathogens can make these infections difficult to treat and presents an urgent medical problem to which there is currently no sustainable solution. However, some individuals seem have a degree of protection against invasion by uropathogenic bacteria (Fouts et al., 2012; Brubaker and Wolfe, 2017; Klein and Hultgren, 2020), as predisposition to UTIs varies greatly between individuals. For instance, the prevalence of recurrent UTI (rUTI) is greatly increased in postmenopausal women (Flores-Mireles et al., 2015; Jung and Brubaker, 2019; Zeng et al., 2022).

Defined in the last decade, the urinary microbiome presents a diverse community of microorganisms, mainly bacteria, that are present at relatively low numbers in the urinary bladder (Wolfe et al., 2012; Ackerman and Underhill, 2017; Wolfe and Brubaker, 2019). Despite the low density of urinary microbes, compositional analyses using culture-independent methods such as 16S rRNA gene sequencing of different human cohorts reveal correlations between this microbiome and multiple urinary health conditions, including urinary urgency, incontinence, cancers, kidney stones, and recurrent UTIs (Fouts et al., 2012; Pearce et al., 2015; Whiteside et al., 2015; Thomas-White et al., 2017; Popović et al., 2018; Govender et al., 2019). Follow-up studies using a more advanced culturing technique—enhanced quantitative urinary culture (EQUC) (Hilt et al., 2014) —confirmed the presence of live bacteria that are typically not identified on a standard urine culture. Similar to the vaginal microbiome, the urinary microbiome often contains lactic acid-forming bacteria including lactobacilli (Thomas-White et al., 2018; Price et al., 2020a). The predominance of lactobacilli in the urinary microbiome or presence of particular lactobacilli species has been associated with protection against UTIs. In particular, some species, such as *Lactobacillus crispatus* and *Lactobacillis iners*, appear to correlate with urinary health (Pearce et al., 2014; Gottschick et al., 2017; Thomas-White et al., 2018; Neugent et al., 2020) but associations with recurrent UTI (rUTI) are less defined. Though a recent study has reported methods for distinguishing lactobacilli at a higher resolution using 16S rRNA gene sequencing, (Hoffman et al., 2021) the majority of urinary microbiome studies, to date, were conducted using an amplicon-based 16S rRNA gene sequencing method that is unable to distinguish lactobacilli at the species or strain levels.

Lactobacilli have been widely used for many years and are generally considered to be safe. Previously characterized lactobacilli, ranging from those used in food preparation to those isolated from the vaginal microbiome, have been investigated for their ability to inhibit the growth of other bacteria. Lactobacilli have been known to inhibit the growth of their competitors by producing organic acids, hydrogen peroxide, surfactants, and special toxins called bacteriocins (Jack et al., 1995; Zacharof and Lovitt, 2012; Mokoena, 2017). Lactobacilli also potentially contribute to probiotic functions. Indeed, many lactobacilli have been studied as potential non-antibiotic treatments to prevent recurrent UTI. However, these studies did not investigate lactobacilli species or strains isolated from the urinary tract (Stapleton et al., 2011; Grin et al., 2013; Gupta et al., 2017; Prabhurajeshwar and Chandrakanth, 2017; Smith et al., 2018; Neugent et al., 2020).

Urinary microbiome properties can provide explanations of observed differences in UTI susceptibility (Wolfe et al., 2012; Brubaker and Wolfe, 2017; Neugent et al., 2020), *via* interaction with the host, *via* the immune response, or *via* intermicrobial interactions. However, only minimal functional data are available for the commensal representatives of the human urinary microbiome. For example, recent examination of urinary isolates of *L. crispatus* found that they use phenyllactic acid to inhibit growth of uropathogens *E. coli* and *E. faecalis* (Abdul-Rahim et al., 2021). This shows similarity of the *L. crispatus* urinary strains with relatives from the vagina as well as diverse food sources (Jacobsen et al., 1999; Valerio et al., 2004; Hütt et al., 2016; Łaniewski and Herbst-Kralovetz, 2021). Additionally, mixed urinary bacterial communities from asymptomatic postmenopausal women and those with acute UTI were shown to interact differently, supporting the idea of complex intermicrobial interactions within the urinary microbiome and potential differential effects on UTI progression (Zandbergen et al., 2021).

We hypothesized that certain urinary lactobacilli will show a strong inhibition to uropathogen growth and thus provide protection against UTI in healthy individuals, while lactobacilli isolated from rUTI patients will not show a strong inhibition. To start addressing this hypothesis, we first collected a representative collection of culturable urinary bacteria from postmenopausal women with and without a history of recurrent UTI. Notably, all isolates were derived from urine in the absence of acute UTI. We then tested the ability of these urinary lactobacilli isolates as representatives of the commensal urinary microbiome to compete with the uropathogens.

MATERIALS AND METHODS

Bacterial Growth and Preservation

Vaughan and coworkers (Vaughan et al., 2021) used published EQUC procedures (Hilt et al., 2014) to isolate urinary bacteria from catheterized urine specimens. Streak-isolated bacteria were typed by MALDI-TOF and then transferred to a set of liquid nutrient media: tryptic soy broth (TSB), Man Rogosa and Sharpe broth (MRS), and brain heard infusion broth (BHI). Inoculated media was statically incubated at 35°C for 24-72 hours with ambient or anaerobic atmospheric conditions. The isolates that did not produce enough biomass were inoculated in TSB supplemented with 5% sheep blood, cysteine (0.5 g/L), or other additives (hemin 5 mg/L, vitamin K 10 mg/L), or NYCIII medium (**Table S1**) and preserved as above if visible biomass was obtained. The qualitative densities of the resulting cultures were recorded and cell cultures were saved in 14% (w/v) glycerol at -80°C in duplicate freezer stocks. Later experiments with preserved strains

showed that growth at 37°C worked similarly to 35°C and was used from then on.

Complete Sanger Sequencing of the 16S rRNA Gene

To confirm species of urinary lactobacilli, we isolated genomic DNA of each used strain from overnight MRS culture using the Qiagen PowerSoil kit. Then we amplified most of the 16S rRNA gene using classic primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') and Q5 polymerase mastermix (NewEngland Biolabs). The resulted PCR product was purified and submitted for Sanger sequencing (Genewiz).

Well-Diffusion Inhibition Assays

Inhibition of a number of substances was measured *via* a well-diffusion assay using solid MRS agar plates. Lactobacilli strains were grown over two nights in 10 mL of MRS broth and uropathogenic strains were grown overnight in 4 mL of LB broth, both stationary at 37°C. Surface of the MRS agar plates was then inoculated with 100 µL of the uropathogenic culture. Wells were punched in the surface (Ø6 mm) and removed with sterile tweezers. 50 µL of the bacterial culture or filtrates to be tested were added to the wells. The plates were then grown overnight at 37°C, unless mentioned otherwise. To measure the size of the zones of inhibition, all plates were first imaged, and the zones were quantified by the graphical program GIMP (www.gimp.org) and analyzed in MS Excel.

Liquid Inhibition Assays

As in the solid agar inhibition assays, lactobacilli strains were grown for two nights in 10 mL of MRS broth and uropathogenic strains were grown overnight in 4 mL of LB broth. The optical density as measured at 600 nm (OD_{600}) of the strains was then taken, and 20 µL of the uropathogen culture was placed into each of the tubes needed for the experiment. The OD_{600} was then used to calculate the amount of lactobacilli culture needed to make specific ratios of uropathogen: lactobacilli and that amount of lactobacilli culture was added to their respective tubes. These tubes were then spun in a centrifuge at 3230 rcf for 10 minutes to form a cell pellet and the supernatant was poured off. The pellets were then resuspended in 10 mL of MRS broth and incubated at 37°C. Measurements of uropathogen density were taken *via* serial dilutions and colony counting and samples were taken at four time points after beginning incubation: 0 hours, 1 hour, 6 hours, and 24 hours. At each of these time points, 100 µL was taken from each tube and used to make serial dilutions ranging from undiluted to a dilution of 10^{-7}. From each of these, 2 µL was taken and plated in rows on LB agar plates which were then incubated for several hours at 37°C. The number of colonies in least diluted but still countable samples was then used to calculate the CFU/mL of *E. coli* in each tube.

Spent Media Manipulations

In several experiments, cells were removed from the culture to isolate the effects of the cells from the effects of cell secretions in the media. This filtrate was made by first spinning a few milliliters of the culture in a centrifuge at 3230 rcf for 10 minutes. The supernatant was then poured into a syringe and pushed through a 0.22 µm PES filter into a sterile tube. This filtrate was then ready for use or further modification as needed for the experiment.

Statistical Analysis

All experiments were done at least in triplicate, with well-diffusion assays being run with technical triplicates on three separate days to represent biological replicates. All p-values were determined by a two-tailed Student's T-test to determine statistical differences between different groups. We used a significance level of 0.05 and p-values of 0.01 or less are marked as well.

RESULTS

Establishing a Repository of Urinary Bacteria

A new urinary bacteria repository was created following the recent study by Vaughan and colleagues (Vaughan et al., 2021). In that study, asymptomatic postmenopausal women provided catheterized urine that was then processed with 16S rRNA amplicon-based gene sequencing and EQUC. Three cohorts of women using vaginal estrogen therapy were tested: those without rUTI [noUTI]; those with rUTI [rUTI]; and those with rUTI and also taking daily prophylactic antibiotics as part of their treatment regimen [rUTI+ab]. Full details on these cohorts including exclusion criteria and demographic information are published elsewhere (see **Tables 1**, **2** of the prior report) (Vaughan et al., 2021). Bacteria isolated using EQUC were typed using mass spectrometry (MALDI-TOF) showing that the collection consists of 64 microbial strains from 24 species of bacteria and two species of fungi (**Tables 1** and **S1**). A subset of lactobacilli was confirmed using Sanger sequencing (**Table S2**). While in 2020 the *Lactobacillus* genus was reclassified into 25 separate genera (Zheng et al., 2020). We have representatives of three of them and will refer to them collectively as lactobacilli throughout the manuscript for simplicity.

Consistent with many urinary microbiome studies, almost half of this collection contains lactobacilli from eight species: *Lactobacillus gasseri, Lactobacillus delbrueckii, Lacticaseibacillus rhamnosus, Ligilactobacillus animalis, Lactobacillus jensenii, Lactobacillus jonsonii, L. iners*, and *L. crispatus*. Due to the nature of the EQUC procedure, all these strains are aerotolerant and our tests showed that the majority of them grow sufficiently well in the presence of oxygen but without active mixing. The exceptions were *L. iners*, some *L. crispatus*, and a few other bacterial isolates which required specialized media or were particularly sensitive to oxygen changes (**Table S1**). Predictably, the rest grew well in MRS broth designed for lactobacilli growth.

It is worth noting that the repository also contains 5 uropathogen species derived in the same fashion from the same research participants: *E. coli, K. pneumoniae*, *K. oxytoca*, *Proteus mirabilis*, and *E. faecalis* (**Tables 1** and **S1**). As all samples were derived in the absence of acute UTI and at least 4 weeks after treatment for UTI (Vaughan et al., 2021), it is unclear if these strains

TABLE 1 | Urinary Isolates repository.

Species	Number Identified
Lactobacilli	*27*
Lactobacillus acidophilus/gasseri	8
Lactobacillus casei/rhamnosus/paracasei	5
Lactobacillus crispatus	5
Lactobacillus delbrueckii	4
Lactobacillus iners	1
Lactobacillus jensenii	2
Lactobacillus spp	2
Strains from well-established uropathogen species	14
Escherichia coli	5
Klebsiella pneumoniae	3
Klebsiella oxytoca	1
Proteus mirabilis	1
Enterococcus faecalis	4
Strains from commensal and opportunistic pathogen species	24
Corynobacerium coyleae	1
Staphylococcus epidermidis	4
Staphylococcus hominis	1
Staphylococcus ludgenensis	1
Streptococcus anginosus	6
Streptococcus bovis group	1
Streptococcus sanguinis alpha	1
Streptococcus sp	1
Aerococcus urinae	3
Leuconostoc sp	1
Raoultella ornithinolytica	1
Diphtheroids	3
Others (fungi)	3
Candida albicans	2
Candida glabrata	1

Identities of isolated microbes in the repository, as determined by MALDI-TOF typing.

were present in the setting of different host immune tolerance mechanisms and if these strains had adapted to survive within the normal urinary microbiome. It is also possible, as recently reported, that similar to the vaginal tract, low level presence of pathogenic bacteria in the bladder is common (Thomas-White et al., 2018; Garretto et al., 2020; Price et al., 2020b).

Two Initial Urinary Lactobacilli Isolates Inhibit Growth of Model Uropathogens

At the beginning of the EQUC collection, we established the whole genome sequences of two initial isolates of urinary lactobacilli – *L. gasseri 5006-2* (GenBank Accession code JAGEKM000000000.1) and *L. delbrueckii 5010-2*. These two strains come from healthy, postmenopausal women and one with a history of rUTI, respectively. Preliminary genome analysis showed the presence of several bacteriocin genes. In light of these preliminary findings, we sought to assess whether major urinary pathogens can be inhibited by these urinary lactobacilli. We used model UTI strains of three major uropathogens: *E. coli, K. pneumoniae*, and *E. faecalis* (**Table 2**) and tested the two sequenced lactobacilli strains for inhibition of pathogens using well-diffusion assays on MRS agar plates (**Figure 1A**).

TABLE 2 | Strains of uropathogen species used in this study.

Species	Strain	Notes/Phenotypes	References/Source
E. coli	MG1655	Non-pathogenic strain	Lab stock
	CFT073	UTI model (urosepsis isolate)	(Mobley et al., 1990); Mehreen Arshad, Northwestern University
	DS17	UTI model (pyelonephritis isolate)	(Tullus et al., 1984); Mehreen Arshad, Northwestern University
	UTI89	UTI model (cystitis isolate)	(Mulvey et al., 2001); Mehreen Arshad, Northwestern University
	ESBL41	MDR clinical isolate	(Kanamori et al., 2017; Bethke et al., 2020); Deverick Anderson, Duke University
	ESBL146	MDR clinical isolate	(Kanamori et al., 2017; Bethke et al., 2020); Deverick Anderson, Duke University
	ESBL168	MDR clinical isolate	(Kanamori et al., 2017; Bethke et al., 2020); Deverick Anderson, Duke University
	ESBL193	MDR clinical isolate	(Kanamori et al., 2017; Bethke et al., 2020); Deverick Anderson, Duke University
K. pneumoniae	TOP52	UTI model (cystitis isolate)	(Rosen et al., 2007; Johnson et al., 2014); Jyl Matson, University of Toledo
E. faecalis	OG1RF	UTI model	Juliett Willett and Gary Dunny, University of Minnesota
	JH2-2	UTI model	Juliett Willett and Gary Dunny, University of Minnesota
	V583	MDR strain	Juliett Willett and Gary Dunny, University of Minnesota
	MMH594	MDR strain	Juliett Willett and Gary Dunny, University of Minnesota

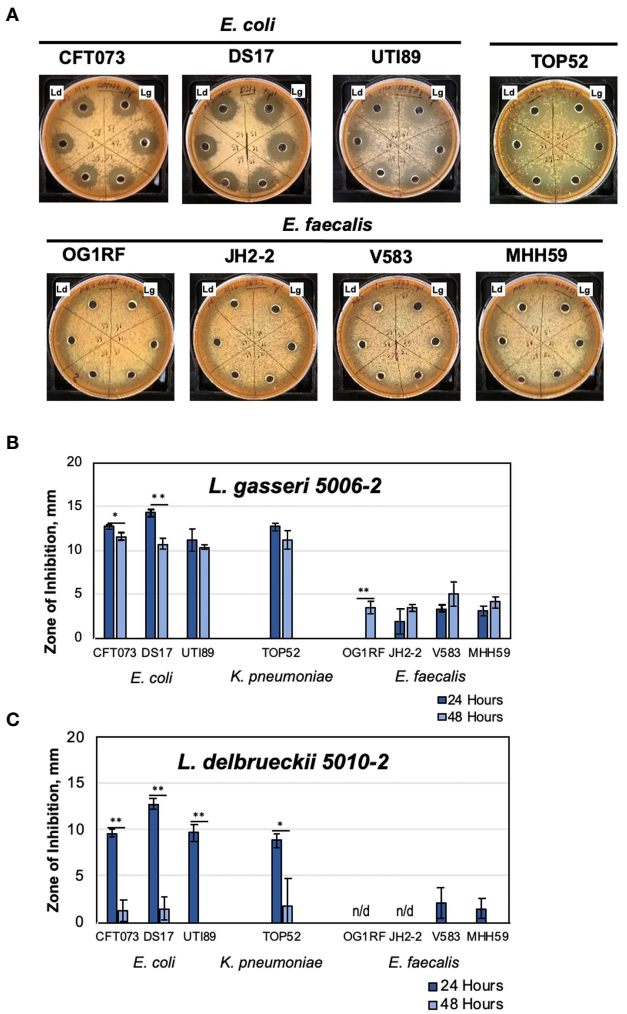

FIGURE 1 | Urinary lactobacilli *L. gasseri* 5006-2 and *L. delbrueckii* 5010-2 inhibit a broad range of uropathogens. Representative images of well-diffusion inhibition assays using *E. coli, K. pneumoniae*, and *E. faecalis* **(A)**, and zone of inhibition sizes of one-day old (dark blue) or two-day old (light blue) cultures of *L. gasseri* 5006-2 [Lg**, (B)**] and *L. delbrueckii* 5010-2 [Ld, **(C)**] against 8 pathogenic strains (**Table 2**). Error bars indicate standard deviation of three biological replicates, *p < 0.05 and **p < 0.01.

Lactobacilli strains were first competed against three different uropathogenic strains of *E. coli*, CFT073, DS17, and UTI89, in a well-diffusion assay (**Figure 1A**) (Tullus et al., 1984; Mobley et al., 1990; Mulvey et al., 2001). Both lactobacilli strains inhibited the growth of all *E. coli* strains, though *L. gasseri* generally showed more inhibition than *L. delbrueckii*. One notable difference between the two strains can be seen in their inhibition when grown for 48 hours, rather than only 24 (**Figures 1B, C**). An *L. gasseri* culture grown for 24 hours seems to have slightly better inhibition of *E. coli* strains than those grown for 48 hours. Cultures of *L. delbrueckii* grown for 24 hours inhibited *E. coli* growth at comparable, albeit slightly lower, levels than the other lactobacilli strain; however, this inhibition dropped massively when cultures were grown for 48 hours, and in some cases nearly to zero. The timing of inhibition appeared to be sensitive to properties of the initial inoculum of *L. delbrueckii* strain.

The inhibition was next tested against other common UTI pathogens – *K. pnemoniae* and *E. faecalis*. For these tests, one strain of *K. pneumoniae* (TOP52) and four strains of *E. faecalis* (OG1RF, JH2-2, V583, and MHH59) were used (**Figures 1B, C**). Significantly, we observed some level of inhibition against all strains tested, even the Gram-positive *E. faecalis* strains (**Figure 1A**). The *K. pneumoniae* strain was sucessfully inhibited by both lactobacilli strains and the amount of inhibition observed was similar to assays performed using *E. coli* UTI model strains. *L. gasseri* cultures grown for both 24 and 48 hours were able to inhibit TOP52. Cultures of *L. delbrueckii* were able to inhibit TOP52 when grown for 24 hours but not when grown for 48 hours. The *E. faecalis* strains were not inhibited nearly as much as the gram-negative strains; however, they were all inhibited to some degree. *L. gasseri* cultures, in particular, were able to inhibit all *E. faecalis* strains to a limited degree after growth for 48 hours and all but OG1RF was inhibited after 24 hours of growth. The *L. delbrueckii* strain, however, was only able to inhibit two of the *E. faecalis* strains at a very low level when grown for 24 hours.

Such inhibition tests were then repeated with antibiotic resistant strains of *E. coli* (ESBL41, ESBL 146, ESBL146, and ESBL193 (Kanamori et al., 2017; Bethke et al., 2020). We determined that lactobacilli inhibition extended to antibiotic resistant *E. coli* as well (**Figure S2**). Notably, tested model *E. faecalis* V583 and MHH95 are also multidrug resistant and can be inhibited by *L. gasseri 5006-2* (**Figure 1**).

Overall, these tests show that the two selected lactobacillus strains were able to consistently inhibit several model uropathogens, potentially indicating a general inhibitory mechanism. These preliminary observations allowed us to set up feasible methods to screen the majority of urinary lactobacilli from our repository under the same growth conditions.

Majority of Tested Strains From Seven Urinary Lactobacilli Species Inhibit the Growth of Model Uropathogens

Our initial tests showed that urinary *L. gasseri 5006-2* and *L. delbrueckii 5010-2* strains can inhibit uropathogens *in vitro*. Moreover, the strength of the inhibition was coincidental with the rUTI status of the patients from whom the strains originated (**Table S3**). We therefore set to test other strains from the same species and five other lactobacilli species in our repository that can be grown under the same conditions. This selection of the lactobacilli species, that were grown under similar conditions, left out several strains of *L. crispatus* and one *L. iners* strain. Overall, we screened 19 lactobacilli strains grown for 24 and 48 hours in static MRS broth. The *Streptococcus anginosus* 5008-3 strain was used as a control for unrelated lactic acid bacteria that are also frequently found in the lower urinary tract (**Table 1**, **S1**). This was done by using a well-diffusion assay against 3 model uropathogens: *E. coli* CFT073, *K. pneumoniae* TOP52, and *E. faecalis* OG1RF (**Figure 2**).

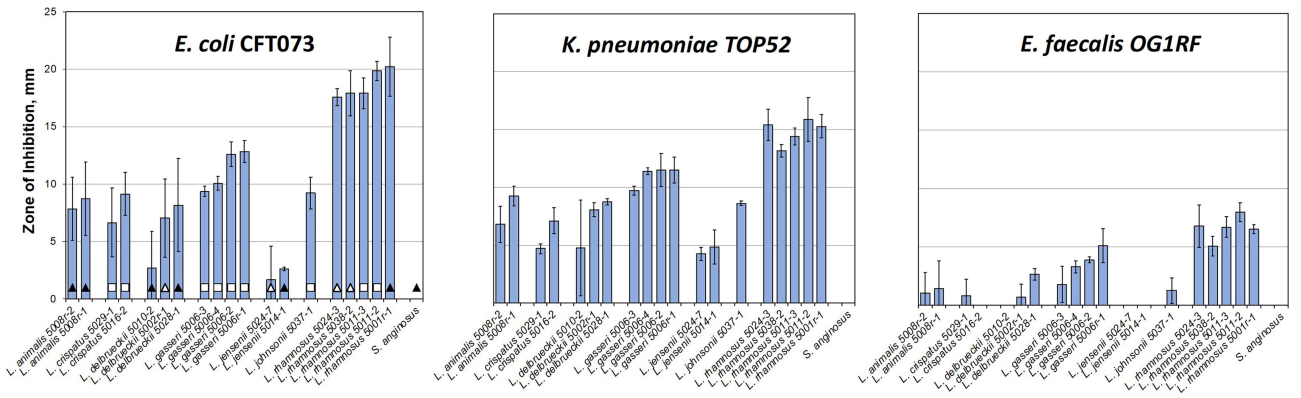

FIGURE 2 | Majority of urinary lactobacilli exhibit varying degrees of inhibition of major uropathogens. Twenty strains of urinary commensal lactic acid bacteria (lactobacilli and one strain of *Streptococcus anginosus* as a control) were tested against model uropathogenic strains: *E. coli* CFT073, *K. pneumoniae* TOP52, and *E. faecalis* OG1RF. Bars indicate the standard deviation of three replicates. The leftmost plot carries identifications of the patient cohorts for each strain (**Table S3**): empty square – no UTI; black triangle – rUTI; white triangle – rUTI with prophylactic antibiotics. The data are presented by species alphabetically and sorted by the inhibition against CFT073 within species set.

Our inhibition data show that the amount of inhibition varied more among different species of lactobacilli than it did among strains within the species. Unfortunately, with only one to five representatives from each species, it is impossible to generalize, apart from recording that the differences between strains and species in our subset are notable. For instance, the *L. rhamnosus* strains all have similarly sized, large zones of inhibition, especially towards the two gram-negative uropathogens. The *L. jensenii* strains did not show a strong inhibition under conditions tested with no detectable inhibition zone on the *E. faecalis* plate. The *L. crispatus*, *L. gasseri*, *L. delbrueckii*, *L. jonsonii*, and *L. animalis* strains all showed intermediate inhibition levels. It is noteworthy that testing 24 and 48 hr. old lactobacilli cultures (**Figure S3**) showed significant differences in inhibition strengths, with *L. crispatus*, *L. delbruecki* and *L. jensenii* being particularly sensitive to lactobacilli culture growth phase.

As the isolates in our repository came from three different cohorts of patients (**Table S2**), we compared the strength of lactobacilli inhibition and cohort status with the hypothesis that there would be greater inhibition in lactobacilli derived from healthy women without rUTI. The presented screen shows that there is no correlation between the patient cohort and the ability of EQUC isolated lactobacilli to inhibit uropathogens *in vitro* (**Figure 2** and **Figure S4**). No statistically significant differences among cohorts are observed for such interspecies or averaged inhibition against either of the three uropathogens (**Figure S4**).

In summary, results show that inhibition is widespread across urinary lactobacilli species, but show heterogeneity among different uropathogens and urinary lactobacilli.

Co-Existence of Antagonistic Species in the Same Bladder

In some of the collected urines, the EQUC method isolated multiple species co-existing in the same sample, including both lactobacilli and uropathogens (**Table S1** and **S3**). As noted, none of the women who provided urine had acute symptomatic UTI and all underwent screening with dipstick urinalysis confirming the absence of pyuria prior to providing research specimens. Nevertheless, the *in vivo* coexistence of uropathogens with lactobacilli species seem to counter the simple hypothesis that presence of uropathogen-inhibiting lactobacilli should eliminate survival of uropathogens in the same niche. For this reason, we sought to test the inhibition of these pathobiont isolates with their cohabitating lactobacilli species to see if lactobacilli that are cohabitating with uropathogens are less antagonistic. To do this, we set up a broad screen to test each of the lactobacilli species isolated from these women (**Table S3**) for inhibition of each of the pathobiont species isolated from that woman's same sample (**Figure S5**). Unfortunately, the *K. pneumoniae* isolates failed to form robust lawns on the test MRS agar plates and therefore, were not included in this cohabitant's screen. Otherwise, all combinations of *E. coli*, *E. faecalis*, and *K. oxytoca* (**Figure S5**) with lactobacilli were measured. To our surprise, most of the measured pairs showed some levels of inhibition, including strain combinations isolated from the same individual at the same time (**Figure 3** and **Table S3**).

We then compared the ability of cohabitating lactobacilli strains to inhibit model uropathogen *E. coli* or *E. faecalis*, strains of the same uropathogen species that coexisted with the given lactobacilli strain, or were isolated from other individuals. From this small subset it appears that the cohabiting *lactobacillus* strain did not inhibit uropathogen isolates as strongly as the inhibition noted previously with model uropathogens (OG1RF and CFT073, **Figure 3A**). With no further genetic or pathogenicity information available for these new isolates, it is not clear if this is due to properties of the lactobacilli or uropathogen strain.

While our data on cross-competing novel clinical isolates against each other, cohabiting or not, it does not support the hypothesis that *in vitro* inhibition can predict *in vivo* behavior – it again shows how the inhibitory interaction is specific and

A

	L. rhamnosus 5024-3	*L. rhamnosus 5011-2*	*L. rhamnosus 5011-3*
E. faecalis 5024-2	5.1 ± 0.7	3.1 ± 2.4	4.4 ± 0.7
E. faecalis 5011-1	4.5 ± 1.7	2.6 ± 1	3.5 ± 2.3
E. faecalis OG1RF	6.8 ±1.8	8 ± 0.8	6.7 ± 0.9
E. coli 5024-1	9 ±1.2	8.8 ± 2.1	9.7 ± 1.1
E. coli CFT073	17.6 ± 0.8	19.9 ± 0.8	17.9 ± 1.3

B

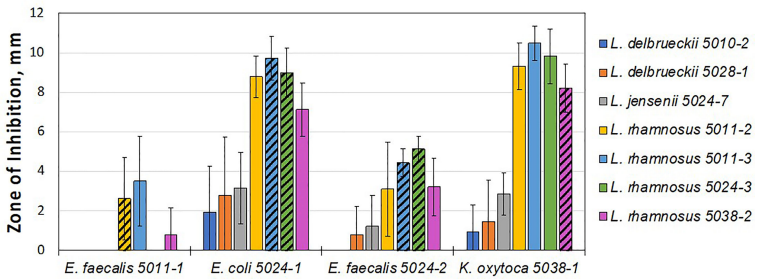

FIGURE 3 | Lactobacilli cohabiting the same bladder with a pathogen still can inhibit that pathogen *in vitro*. **(A)** Table showing average zones of inhibition (mm) by cohabiting *E. coli* and *E. faecalis* strains. The comparison indicates that cohabiting pathogen strains (shaded in grey) are inhibited by the respective cohabiting commensal urinary lactobacilli but to lesser degree than other pathogen isolates and the model uropathogen strains (no grey shading). **(B)** Overall screen of patient isolates shows that 'better inhibiting' lactobacilli strains against one pathogen isolate also show 'better inhibiting' against another pathogen isolate. In addition, *L. rhamnosus* strains appear to be better inhibitors, similar to the model screen (**Figure 2**).

sensitive to the identity of the lactobacilli used at a strain-level. Nevertheless, we do observe that some species tend to be better inhibitors on a relative scale, when compared to others. Specifically, *L. delbrueckii 5010-2, L. delbrueckii 5028-1*, and *L. jensenii 5024-7* did not inhibit well in a screen with model uropathogens and they consistently exhibit less inhibition with uropathogen isolates derived clinically from patients (compare **Figures 1**-**3**).

The results of testing urinary lactobacilli and pathobionts found in the same urine sample indicate that antagonistic species might coexist *in vivo*.

Interference of Urinary Lactobacilli With Uropathogens in Liquid Media

While informative, simple inhibition on agar plates is not be reflective of the physiological conditions of the lower urinary tract. Therefore, we tested the interactions of urinary lactobacilli and UPEC in liquid MRS media, which might provide a better, though still not perfect, representation of bacterial suspension in urine within the lower urinary tract. We therefore co-cultured *E. coli* CFT073 with increasing amounts of *L. gasseri 5006-2* or *L. delbrueckii 5010-2* in liquid growth media.

The presence of either of these lactobacilli strains dramatically decreased the growth of the *E. coli* CFT073 in media (**Figure 4**). This can be seen in the significantly reduced amount of *E. coli* after only 6 hours of growth together with either strain of lactobacilli. After 24 hours, both *L. delbrueckii 5010-2* and *L. gasseri 5006-2* were able to reduce CFT073 densities by two to five orders of magnitude. Cultures containing the *L. gasseri 5006-2* strain, similar to results in the well-diffusion assays, were able to inhibit to a greater degree than *L. delbrueckii 5010-2* cultures.

These experiments showed that the dynamics and strength of inhibition in liquid is different between the lactobacilli strains.

Mechanism of Uropathogen Inhibition by Select Lactobacillus Species

To determine how the inhibition took place, lactobacilli *L. gasseri 5006-2* and *L. delbrueckii 5010-2* cultures were split into two parts: a cell-free filtrate and a suspension of cells in fresh broth. The filtrate of both lactobacilli cultures could inhibit the growth of the *E. coli* CFT073 strain starting after about 12 hours of incubation together on a plate and lasting until about 24 hours of incubation together (**Figure 5**). However, this inhibition seems to be somewhat transient as when plates were incubated for longer than 24 hours, the *E. coli* would begin overgrowing the zone of inhibition, causing the clearance zone to shrink until it vanished in ~ 4 days (**Figure S6**). Zones from the resuspended cells, in contrast, differed markedly between the two lactobacilli cultures. Resuspended *L. delbrueckii 5010-2* cells exhibited some inhibition, but usually not as strong as that of cell-free filtrate or *L. gasseri 5006-2* strain (**Figure 5**). Resuspended *L. gasseri 5006-2* cells showed inhibition comparable to that of the respective whole culture. In addition, the zones of inhibition from resuspended cells of both strains did not change if the plates were incubated longer than the standard 24 hours while filtrate-induced inhibition zones were eventually overgrown by *E. coli* lawn (**Figure S6**). This seems to indicate an additional mechanism of inhibition present in the *L. gasseri* cultures; one that is dependent on the presence of cells and which does not seem to be resulting from acidification. This seems to show that both lactobacilli species inhibit uropathogens by decreasing the pH of their environment and the *L. gasseri* strain may have a

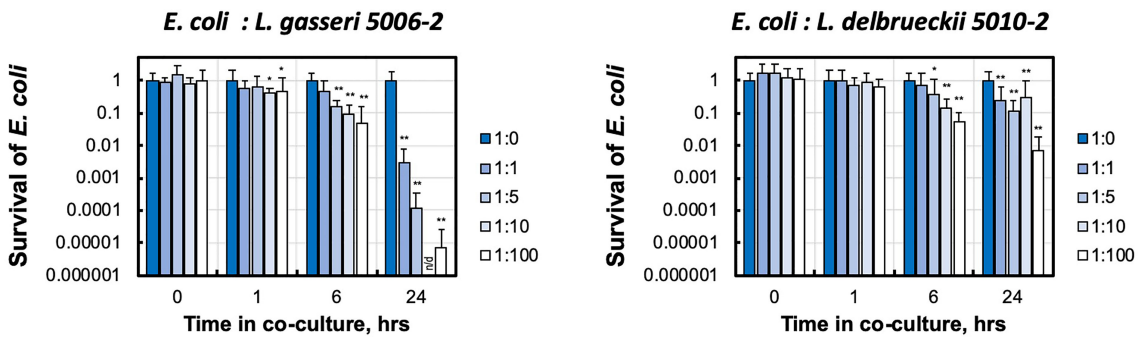

FIGURE 4 | Urinary lactobacillus strains inhibit *E. coli* growth when co-cultured in liquid medium. Uropathogenic *E. coli* CFT073 was grown in presence of increasing amounts of initial lactobacilli (*L. gasseri* 5006-2 and *L. delbrueckii* 5010-2) with ratios 1:0, 1:1, 1:5, 1:10, and 1:100 estimated by the OD600 measurements. *E. coli* survival was measured by LB plating of serial dilutions for CFU counting and compared with growth of *E. coli* only culture (1:0). Survival is calculated as culture density normalized to *E. coli* only culture. Error bars show only positive arm of standard deviation for three biological replicates. *p < 0.05 and **p < 0.01 and indicate a significant decrease from *E. coli* grown in the presence of lactobacilli. Over the course of this experiment *E. coli* for lactobacilli-free condition (1:0) grows from ~ 2.5-3.4×10^6 to ~ 8.4 - 9.7×10^8 CFU/mL densities. n/d – the limit of detection of our CFU counting was at about 500 CFU/mL, corresponding to ~ 0.5×10^{-6} survival.

strong additional cell-based mechanism of action that is independent of a low pH.

The cell-free filtrates from both *L. delbrueckii* and *L. gasseri* cultures were then heated or adjusted to a neutral pH before use in a well-diffusion assay. The filtrate showed the same amount of inhibition when heated as it did when unmodified, indicating that the mechanism of inhibition is resistant to heat. The filtrate from both cultures that was adjusted to a neutral pH, conversely, showed no inhibition. In addition to neutralizing the filtrate, another experiment increased the buffering capacity of the medium used in the experiment by adding PBS to the MRS agar formulation. These plates showed significantly reduced inhibition by filtrates from both lactobacilli cultures. Finally, pH readings of the filtrate before use in well-diffusion assays seem to indicate that a low pH (~3.9-4.0) is needed for inhibition to occur. Taken together, this seems to indicate that the mechanism of inhibition in the cell-free filtrate is highly dependent on pH and is likely a result of the acidification of their environment by lactobacilli species.

One possible explanation for cell-dependent inhibition is the presence of biosurfactants. Several reports showed that phosphate-buffers can elute such surfactants into solution (Rodrigues et al., 2006; Gudiña et al., 2011; Morais et al., 2017). To test for this hypothesis, cells were incubated in PBS for varying lengths of time (1-24 hr) to isolate any biosurfactant molecules, then the PBS solution was filter-sterilized, and used in well-diffusion assays. However, the PBS incubated with either of the strains' cultures was not able to inhibit CFT073, indicating a lack of inhibitory biosurfactants that can be eluted this way (*data not shown*).

DISCUSSION

In this study, we conducted initial functional characterization of urinary lactobacilli from seven species under uniform conditions. Three of those species (eight strains) are from the abundant and frequently identified species in the female urinary tract: *L. crispatus, L. gasseri* and *L. jensenii* (Ma et al., 2012; Komesu et al., 2020; Neugent et al., 2022). While lactobacilli inhabiting other ecological niches have been previously studied, urinary lactobacilli have not previously been characterized, despite their potential for direct interactions with uropathogens and potential clinical implications regarding urinary tract infections.

We found that most urinary lactobacilli species strongly inhibit the growth of gram-negative *E. coli* and *K. pneumoniae*, including model strains and drug-resistant clinical isolates. Many of these same strains, however, either showed modest levels of inhibition or did not inhibit the growth of the gram-positive *E. faecalis*. *L. gasseri 5006-2* and *L. delbrueckii 5010-2* inhibited the growth of uropathogenic *E. coli* both on solid agar and in liquid media (**Figures 1**–**4**). Such inhibition is relevant to bacterial interaction at the urothelial surface and in bulk urine, respectively.

Our study shows that there is high variability of inhibition strength among lactobacilli species and sometimes strains of the same species. The data for *L. gasseri* and *L. rhamnosus* species identifies them as strong inhibitors of uropathogens *in vitro*. *L. delbrueckii* is a less efficient inhibitor. The *L. jensenii* and *L. crispatus* strains tested show only modest inhibition in our assays.

Interestingly, *L. crispatus* has been previously associated with a healthy host urinary tract in some urinary microbiome studies (Pearce et al., 2014; Neugent et al., 2022) but no difference in abundance of this species was found in others (Komesu et al., 2020; Vaughan et al., 2021). Urinary *L. crispatus* isolates recently were found to inhibit *E. coli* CFT073 and *E. faecalis* in TSB medium *via* accumulation of phenyl-lactic acid (Abdul-Rahim et al., 2021). On the contrary, the urinary strain of *L. gasseri UMB4205* tested by Abdul-Rahim and colleagues was not inhibiting *E. coli* growth on TSB agar (Abdul-Rahim et al., 2021). These differences may confirm the overall heterogeneity in the urinary lactobacilli phenotypes we observed or may reflect on the sensitivity to the culture testing conditions among the studies. It is also plausible that different phenotypes are

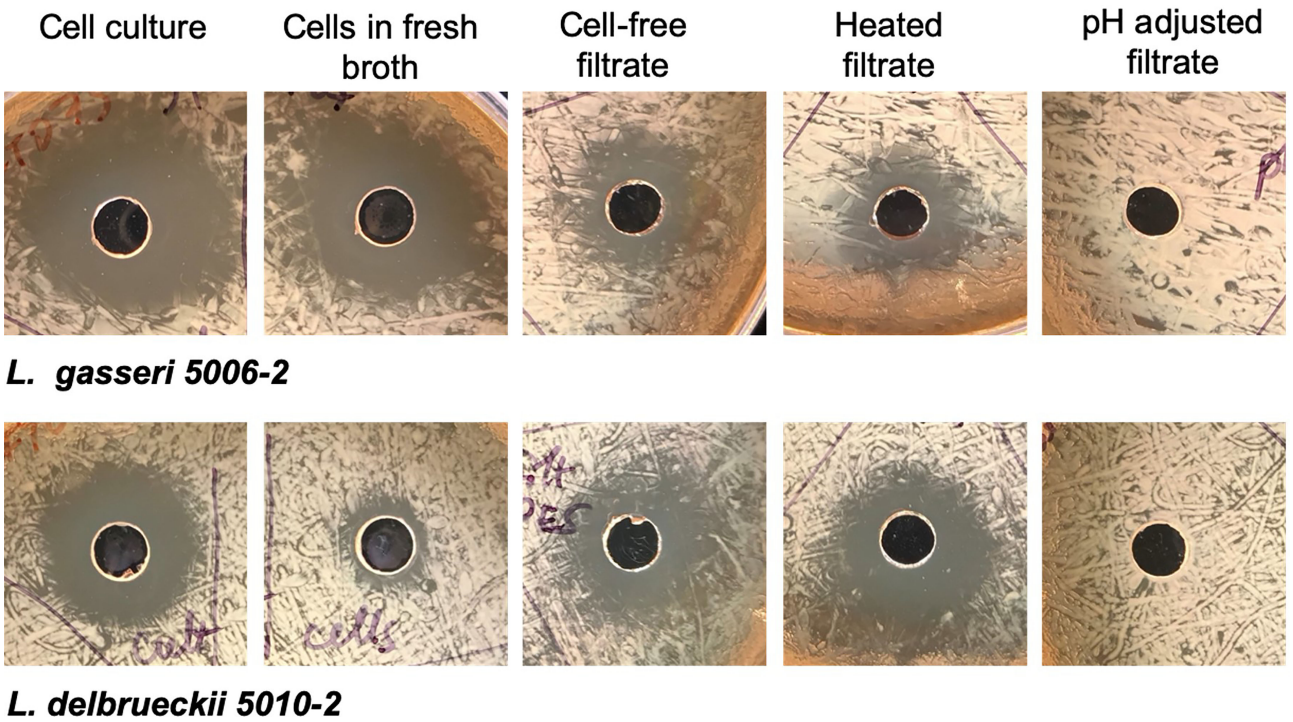

FIGURE 5 | Lactobacilli inhibition differs depending on culture treatment. Representative images from well-diffusion assays using whole cell culture, cells resuspended in fresh broth, cell-free filtrate, heated filtrate, and filtrate adjusted to a neutral pH. Images taken for 24 hrs. growth at 37°C.

characteristic if isolates are derived from the urinary bladder in different age groups or health conditions and thus, findings from individual species may not be comparable 'across the board'.

Similar heterogeneity in phenotypes was previously found for vaginal lactobacilli. For example, vaginal *L. crispatus* isolates were shown to be stronger inhibitors of *E. coli* and candida than *L. jensenii* and *L. gasseri* in a study by (Hütt et al., 2016). Other screening of vaginal *L. gasseri* and *L. crispatus* showed strong differences in some antimicrobial activities, but not others (Atassi et al., 2019).

The inhibition heterogeneity and additional testing of the two isolates (*L. gasseri* and *L. delbrueckii*) suggest that the underlying inhibition mechanisms are complex. At least for *L. gasseri* 5006-2, inhibition has both pH-dependent and cell-dependent components (**Figure 5**). Acidification of the environment by abundant lactobacilli is thought to be the main factor resisting pathogens in the vagina. However, in contrast to the vagina, the bladder microbiome cannot have overall high lactobacilli density. In addition, bulk urine pH is changing with metabolism and diet making it unlikely to maintain an overall low urine pH. Nevertheless, it is possible that in some niches where lactobacilli adhere, they can cause local acidification and thus, adjacent protection against uropathogens.

While it is tempting to look for simple differences in binary inhibitory interactions of urinary lactobacilli with uropathogens to explain propensity towards rUTI, our data did not show correlations between strength of lactobacilli inhibition and patient cohorts (**Figure 2** and **S4**). This is consistent with the lack of strong correlations in postmenopausal urinary microbiome composition with rUTI status (Vaughan et al., 2021). Moreover, the observed inhibition of lactobacilli against the growth of pathogens found in the same urine samples rules out the hypothesis that inhibition of a uropathogen by present lactobacilli will automatically eliminate the uropathogen. These observations are not entirely unexpected, as lactobacilli and uropathogen are only a part of a complex urinary microbiome in which presence of other bacteria might assist or negate the inhibitory lactobacilli effects.

Even binary interaction between lactobacilli and *E. coli* uropathogen might include aspects other that inhibition by lactobacilli. For example, there is a known example of an interaction in which presence of uropathogens might enhance the survival of auxotrophic lactobacilli, similar to those found in the bladder, in minimal medium M9 (Mizuno et al., 2017). *E. coli* was also found to co-aggregate with several lactobacilli (Mizuno et al., 2014). In addition, adhesion of lactobacilli to gut and vaginal epithelium was shown to inhibit pathogen adhesion (Osset et al., 2001; Otero and Elena Nader-Macías, 2007; Wang et al., 2018; He et al., 2020) and such effects can be applicable to the urinary tract.

While we tested several isolates of lactobacilli, it possible that any isolates from postmenopausal women are less protective *in*

vivo against UTI, potentially explaining the documented susceptibility of this patient population to rUTI (Pearce et al., 2014; Komesu et al., 2020; Vaughan et al., 2021; Neugent et al., 2022; Zeng et al., 2022). Therefore, to advance our understanding of the urinary microbiome interactions with uropathogens, it will be essential to compare the behavior of urinary lactobacilli from healthy and rUTI cohorts of pre-menopausal women. Nevertheless, the presented characterization of urinary lactobacilli here, from postmenopausal women with and without rUTIs, is critical to our understanding of how lactobacilli function within the postmenopausal urinary tract. These findings also provide inferences for which lactobacilli probiotic candidates can stably colonize the niche.

In liquid inhibition, the results indicated that the ratio between the uropathogen and commensal is important for efficient inhibition over time (**Figure 4**). Thus, the future studies need to identity uropathogen(s) in a given rUTI patient to establish densities and distribution of the lactobacilli and uropathogen cells and to characterize the dynamics of this complex community. Without the detailed data, we cannot distinguish situations in which rUTI patients may continue to remain asymptomatic due to urinary lactobacilli, keeping uropathogen populations 'in check' or in which urinary lactobacilli simply cannot inhibit the growth of the uropathogens.

In conclusion, our study shows that urinary lactobacilli behave like lactobacilli isolated from other sources and exhibit the strong ability to inhibit the growth of different uropathogens. This inhibition is not limited to classic acidification of media and broadly differs among the species and strains inhabiting the bladder, uropathogen type, and conditions of the lactobacilli growth. These results highlight the necessity of detailed investigations of the composition of the urinary microbiome at the species and sub-species resolution level and careful functional characterization of urinary microbial representatives, separately and in communities. These studies will bring us closer to understanding UTI progression and recurrence and to developing UTI-targeting personalized pre- and probiotics.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

Conceptualization and method development: TS. Experimental design and performance: JJ, TS, MR, VO, LD, and KH. Repository creation: NS and TS. Data analysis: JJ and TS. Funding acquisition and project management: TS. Manuscript writing and editing: JJ, NS, and TS. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.870603/full#supplementary-material

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Updating Urinary Microbiome Analyses to Enhance Biologic Interpretation

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Objective: An approach for assessing the urinary microbiome is 16S rRNA gene sequencing, where analysis methods are rapidly evolving. This re-analysis of an existing dataset aimed to determine whether updated bioinformatic and statistical techniques affect clinical inferences.

Methods: A prior study compared the urinary microbiome in 123 women with mixed urinary incontinence (MUI) and 84 controls. We obtained unprocessed sequencing data from multiple variable regions, processed operational taxonomic unit (OTU) tables from the original analysis, and de-identified clinical data. We re-processed sequencing data with DADA2 to generate amplicon sequence variant (ASV) tables. Taxa from ASV tables were compared to the original OTU tables; taxa from different variable regions after updated processing were also compared. Bayesian graphical compositional regression (BGCR) was used to test for associations between microbial compositions and clinical phenotypes (e.g., MUI versus control) while adjusting for clinical covariates. Several techniques were used to cluster samples into microbial communities. Multivariable regression was used to test for associations between microbial communities and MUI, again while adjusting for potentially confounding variables.

Results: Of taxa identified through updated bioinformatic processing, only 40% were identified originally, though taxa identified through both methods represented >99% of the sequencing data in terms of relative abundance. Different 16S rRNA gene regions resulted in different recovered taxa. With BGCR analysis, there was a low (33.7%) probability of an association between overall microbial compositions and clinical phenotype. However, when microbial data are clustered into bacterial communities, we confirmed that bacterial communities are associated with MUI. Contrary to the originally published analysis, we did not identify different associations by age group, which may be due to the incorporation of different covariates in statistical models.

Conclusions: Updated bioinformatic processing techniques recover different taxa compared to earlier techniques, though most of these differences exist in low abundance taxa that occupy a small proportion of the overall microbiome. While overall microbial compositions are not associated with MUI, we confirmed associations between certain communities of bacteria and MUI. Incorporation of several covariates that are associated with the urinary microbiome improved inferences when assessing for associations between bacterial communities and MUI in multivariable models.

Keywords: urinary microbiome, urobiome, bioinformatic analysis, mixed urinary incontinence, bladder dysfunction, lactobacilli, microbiota

INTRODUCTION:

The urinary microbiome is being investigated in multiple bladder conditions. There are now several reports demonstrating differences in urinary microbiota in women with recurrent urinary tract infection (UTI) (Burnett et al., 2021; Vaughan et al., 2021), urgency urinary incontinence (Pearce et al., 2014; Karstens et al., 2016), and mixed urinary incontinence (Komesu et al., 2018) when compared to matched controls without these symptoms. In most of these studies, 16S rRNA amplicon sequencing has been employed as a culture-independent method of detecting urinary bacteria. When using sequencing to detect bacteria, DNA is extracted from a biological sample, polymerase chain reaction (PCR) is used to amplify and sequence portions of the 16S rRNA gene, and bioinformatic tools are used to match the recovered sequences with those existing in a reference database. Results are reported as taxonomic groupings (i.e., taxa). These steps allow investigators to identify the bacteria contained in a sample. Next, the recovered taxa can be compared between participant cohorts using statistical analyses to discern if there are differences between phenotypic groups.

The bioinformatic steps outlined above depend on multiple computational components, which have been rapidly evolving. Many prior analyses were performed with reference databases that have not been recently updated, such as Greengenes[1]. Furthermore, the Greengenes database does not have substantial representation of urinary bacteria and thus may not be the optimal reference database for identification of microbiota within a urine sample (Hoffman et al., 2021). Regardless of the reference database that is selected, bioinformatic workflows rely on algorithms that group raw sequencing data based on similarities (Knight et al., 2018). These algorithms are rapidly evolving, and when updated or refined, could potentially alter bacterial identification results (Kopylova et al., 2016; Callahan et al., 2017; Edgar, 2017; Nearing et al., 2018; Caruso et al., 2019). Previously, researchers would group raw sequences into operational taxonomic units (OTUs) based on similarity, then compared these OTUs against reference databases to identify the bacterial taxa (Westcott and Schloss, 2015). Many now advocate for grouping raw sequences using amplicon sequence variant (ASV)-based methods, where sequences are grouped based on their error-corrected exact sequences (Callahan et al., 2017). In ASV-based methods, the error score assessing the confidence of sequencing results at each nucleotide is incorporated such that algorithms can better detect true biologic sequences versus those generated by sequencing error. Furthermore, ASV-based methods have the ability to identify bacterial taxa at finer resolution (e.g., genus and species levels where previously identifications were at higher taxonomic levels such as the family level). Studies that were performed prior to these updates in bioinformatic workflows may benefit from re-analysis.

Separate from bioinformatic components of analyses, the statistical methods used to analyze microbial data are also evolving. Prior studies have used methods such as Dirichlet Multinomial Mixture (DMM) modeling (Holmes et al., 2012), which adopt simplistic distributional assumptions on the microbiome compositions, and linear discriminant analysis effect size (LeFSE)[2], which is a nonparametric cross-sample test that utilizes linear discriminant analysis (LDA) to construct test statistics assisted by classical univariate tests for feature (e.g., organism, clade, or OTU) selection. Neither approach adequately accounts for all of the key characteristics of microbiome data such as their compositional constraints, complex cross-sample heterogeneity, and sparse counts of certain taxa. Thus, high dimensional microbial datasets that are used in these types of compositional or community-based analyses fail to meet the underlying assumptions that are needed for the statistical techniques. Rather, more recently developed tree-based models (Wang and Zhao, 2017; Mao et al., 2020), community-based analyses (Layeghifard et al., 2017), or other models that more truthfully account for the distributional characteristics may be needed, especially in view of the limited sample sizes in most studies. These modeling techniques are currently under further development and may be able to better detect the true signal within a dataset.

For datasets with robust findings, updated analytic techniques should not substantially alter major findings. However, urinary microbiome results could be especially prone to bias or skew from different analytic techniques, since small differences are magnified in low biomass environments (Caruso et al., 2019). We hypothesized that updated analyses would enhance precision and allow for more clarity with biologic inferences and thus we

[1] https://greengenes.secondgenome.com/

[2] https://twbattaglia.gitbooks.io/introduction-to-qiime/content/lefse.html

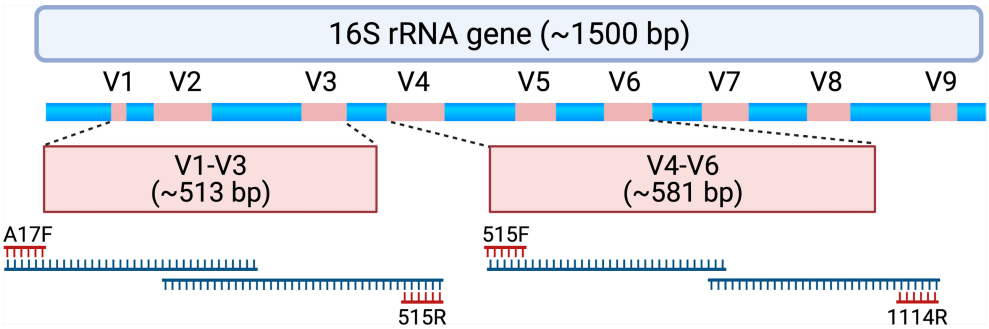

FIGURE 1 | Schematic depicting the 16S rRNA gene and approximate locations of variable regions (V1-V9) that can be selected for amplicon-based sequencing. Samples assessed for this study underwent polymerase chain reaction (PCR) to amplify the V1-V3 region using PCR primers A17F and 515R. When forward and reverse reads are merged, the entire V1-V3 region spans 513 ± 22 base pairs with approximately 87 base pairs of overlapping sequence. Samples also underwent PCR to amplify the V4-V6 region using PCR primers 515F and 1114R. When forward and reverse reads are merged, the entire V4-V6 region spans 581 ± 2 base pairs with approximately 19 base pairs of overlapping sequence. For this study, forward and reverse reads were generated on an Illumina MiSeq platform, which creates sequencing reads of approximately 300 base pairs in length. The initial and final portions of each sequencing read tend to contain lower quality sequence (i.e., lower confidence scores with nucleotide assignment) that could be adjusted or truncated in a DADA2 processing pipeline. As such, paired end reads without a substantial amount of overlapping sequencing may not be able to be merged. Created with BioRender.com.

used updated techniques to re-analyze raw sequencing data generated in a prior study (Komesu et al., 2018). Our primary objective was to determine whether taxonomic identifications substantially differ with an updated bioinformatic pipeline. Secondary objectives were to compare taxonomic identifications based on the 16S rRNA gene variable region used, to assess whether tree-based modeling strategies enhance our ability to differentiate microbial community profiles between women with mixed urinary incontinence and controls, and re-assess relationships between bacterial communities and mixed urinary incontinence with updated information.

METHODS

After Duke University Institutional Review Board approval (Pro #00102155), we conducted a re-analysis of sequencing data generated from the Human Microbiome Study in the Effects of Surgical Treatment Enhanced with Exercise for Mixed Urinary Incontinence HMS-ESTEEM Study (Komesu et al., 2018). The HMS-ESTEEM study was a supplemental translational study embedded within the ESTEEM randomized trial (Sung et al., 2019) conducted by 8 clinical sites within the Pelvic Floor Disorders Network (PFDN)[3]. Briefly, this was a cross-sectional analysis of microbiome data obtained from women with mixed urinary incontinence (MUI) and age-matched controls. The strict inclusion and exclusion criteria for participants (207 women, 123 with MUI and 84 age-matched controls) are detailed in prior publications (Sung et al., 2016; Komesu et al., 2017). Women completed validated questionnaires to assess urinary symptom burden and to confirm appropriate categorization into MUI and control groups. Additional questionnaires were administered to gather data about hormonal therapies, sexual activity, recent infections, and the presence of any vaginal medications. Urine samples were obtained *via* transurethral catheterization and stored in a DNA protectant (Assay Assure™, Sierra Molecular Corporation, Incline Village, NV, USA). Samples were transferred with cold packs *via* overnight shipping to a central laboratory where they were immediately processed, and DNA was extracted. DNA was stored at -80°C prior to sequencing until all samples were collected. DNA was then thawed, subjected to polymerase chain reaction (PCR) amplification and 16S rRNA gene sequencing. For each sample, two separate 16S rRNA gene regions (i.e., the V1-V3 and V4-V6 hypervariable regions) were sequenced (**Figure 1**). Laboratory methods, primers, and details regarding a multi-step PCR (total 38 cycles) are described in detail in a methodology paper associated with the original study (Komesu et al., 2017).

Bioinformatics

We obtained unprocessed sequencing files from the Sequence Read Archive[4] Bioproject #703967 previously generated OTU tables, and associated clinical data from the PFDN data coordinating center. First, we repeated sequence processing using updated techniques. Differences between original and updated processing are illustrated in **Figure 2**. In the original analysis, sequencing data were processed using the Illumina BaseSpace 16S Metagenomics App version 1.0.1. This software classifies raw sequencing data using ClassifyReads, a high-performance implementation of the Ribosomal Database Project (RDP) classifier (Wang et al., 2007), and compares classified sequence reads against the Greengenes reference database to identify bacteria. The output is an OTU table, which is a designation of relative proportions of different taxonomic groups that each sample contains. In the updated

[3]https://pfdnetwork.azurewebsites.net/

[4]https://www.ncbi.nlm.nih.gov/sra

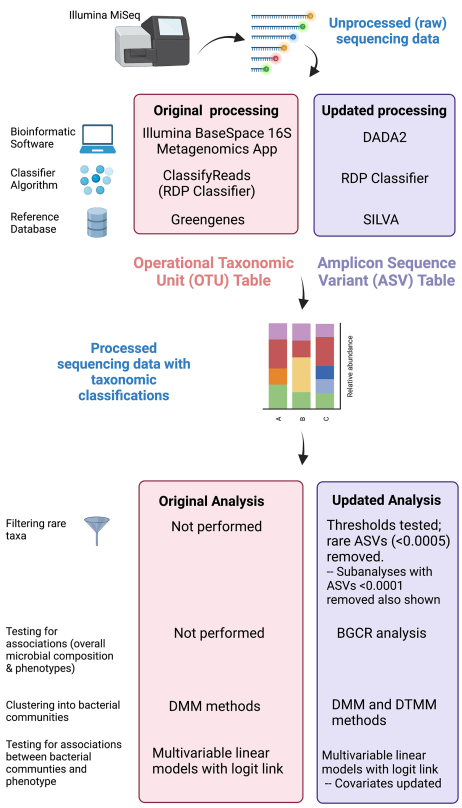

FIGURE 2 | Comparison of original and updated methods. Unprocessed sequencing data generated from an Illumina MiSeq platform were obtained. The figure illustrates differences in the bioinformatic software, classifying algorithm, and reference databases between original and updated processing. The output from original processing was an OTU table. The output from updated processing was an ASV table, which is similar to an OTU table but also adjusts for sequencing errors. Both tabular outputs can be incorporated into Phyloseq objects in R to visualize data as stacked bar plots. Original and updated statistical analysis methods including filtering, clustering, and methods used to test for associations are also listed. Created with BioRender.com.

analysis, raw sequences files were processed using the learnErrors and derepFastq functions in DADA2 (Callahan et al., 2016)(v 1.14.0) with default parameters, then mapped to the SILVA reference database (Quast et al., 2013)(v 132) with the RDP classifier implemented in the assignTaxonomy function in DADA2. The end result is an ASV table, which is similar to an OTU table but takes into account sequencing error rather than sequence similarity when grouping sequences together. Data were further processed and visualized in R using phyloseq (McMurdie and Holmes, 2013) (v. 1.26.1) and microshades (Dahl et al., 2021) (v. 0.0.0.9).

Comparisons of Recovered Taxa in Original Versus Updated Analysis

Using phyloseq and microshades in R, plots were generated to illustrate the recovered taxa identified in the original analysis (i.e., OTU table) and updated analysis (i.e., ASV table). Notably, these comparisons were performed on unfiltered data. Relative abundances of recovered taxa per sample were calculated for original and updated analyses, and directly compared to assess for differences in recovered taxa based on bioinformatic processing.

Comparisons of Recovered Taxa by Sequencing Amplicons

When performing 16S rRNA gene sequencing, generally one or more variable regions (commonly V1-3 or V4-6) of the 16S rRNA gene are amplified and sequenced; the variable regions chosen are referred to as amplicons. Different amplicons contain distinct regions of DNA, vary in length, and may have different representation in reference databases. As such, one amplicon may identify a specific bacterium at higher resolutions or in a more specific manner than another amplicon targeting a different variable region. Each DNA sample in this study was subjected to sequencing using two different amplicons, one targeting the V1-V3 variable region and the other targeting the V4-V6 variable region. We compared taxa identified from the same DNA sample using both amplicons to understand which taxa might be differentially identified in urine based on the amplicon chosen for sequencing. Data were visualized in R using phyloseq and microshades. Plots were generated to illustrate the recovered taxa in samples with paired V1-V3 and V4-V6 amplicon data. Relative abundances of recovered taxa per sample were calculated for each amplicon and were compared between amplicons.

Rare ASVs: Filtering Thresholds

ASV tables generated from the updated bioinformatic analysis were incorporated into downstream statistical analyses, including clustering to assess for latent community structure. Notably, in the original study, DMM clustering was performed on unfiltered data. However, given the low biomass sample type and potential for contaminants to influence results, we chose to filter data prior to clustering. Data were filtered in R based on the relative abundance of read counts with taxa below a specified threshold removed from the dataset. We used a hybrid of the "Rule of Thumb" and "Statistical Threshold" methods that have been previously reported (Cao et al., 2020). After initial review, the microbial data contained within this dataset were mainly dominated by a few ASVs with many other ASVs having low relative abundances. Thus, we assessed a range of filtering thresholds from 0.05 - 0.0001 and the subsequent effects on downstream clustering for the entire dataset (i.e., prior to assigning MUI versus control labels). Coarse filtering thresholds of 0.05 – 0.001 resulted in removal of some low abundance taxa (including *Escherichia/Shigella*) that have been demonstrated in other urinary microbiome studies and are inferred as being true representatives of the urinary microbiome. Furthermore, when using coarse thresholds, we observed non-informative clusters that did not appear biologically distinct. We selected a more conservative filtering threshold of 0.0005, though in several places we illustrate how a less restrictive threshold of 0.0001, which more closely

approximates unfiltered data, might have influenced downstream results.

Testing for Associations Between Individual Taxa and Clinical Phenotypes

In the original study by Komesu *et al.* (Komesu et al., 2018), the primary aim focused on differences in *Lactobacillus* predominance between clinical phenotypes (i.e., MUI and control). However, there were no methods employed to determine whether other specific taxa are associated with MUI vs. control. Bayesian graphical compositional regression (BGCR) is a technique that allows us to test for associations of individual taxa (including rare taxa) with outcomes (Mao et al., 2020). BGCR models the distribution of microbiome data while incorporating phylogenetic relationships and adjusts for other variables that could potentially confound associations with outcomes. BGCR inherently controls for multiple testing and returns a posterior joint alternative probability (PJAP) with a larger PJAP (closer to 1) indicating evidence of differences in taxa between groups. We tested for differences in taxa between MUI and control participants using this technique. BGCR (Mao et al., 2020) was performed in R using BGCR v0.1.0. For BGCR analyses we included the same covariates that we later included in models assessing community structure and phenotypes. These were: age, smoking status, ethnicity, body mass index (BMI), composite menopausal/hormonal status, vaginal pH, history of recurrent UTI, and number days from the most recent catheterization. The strategy behind variable selection is further discussed below and in **Supplemental Table 1**.

Clustering Into Bacterial Communities

Typically, a microenvironment (e.g., urinary bladder) will contain several taxa that are considered together as a bacterial community. While some conditions might have unique bacterial taxa associated a phenotype, in other conditions, there may be overarching differences in bacterial community structure that are associated with phenotypes. Thus, when trying to infer clinical implications from microbial datasets, samples are often clustered based on those that contain similar combinations of taxa, resulting in a substructure of several bacterial communities. Bacterial communities (rather than individual taxa) can then be assessed for associations with clinical phenotypes. In the original analysis, unfiltered taxa were clustered into bacterial communities using Dirichlet Multinomial Mixture (DMM) (Holmes et al., 2012), and these communities were subsequently tested for associations with MUI vs. control phenotypes. In DMM methods, investigators assign the number of clusters (corresponding to bacterial communities) that are desired. This is achieved by reviewing results with different numbers of clusters and selecting the final number of clusters that qualitatively seems to make sense. We repeated DMM clustering on ASV data from the updated bioinformatic analysis in R using DirichletMultinomial v 1.36.0. We chose the same number of clusters that were selected in the original publication. However, since selecting the number of clusters can introduce bias, we also evaluated another method of clustering that automatically adapts the number of clusters based on the complexity of the data. This is a nonparametric mixture model that utilizes the phylogenetic tree to enrich the modeling on cross-sample variability called Dirichlet tree multinomial mixture (DTMM) (Mao and Ma). We performed this clustering method in R using DTMM v0.1.0[5]. While DMM clustering is highly influenced by the "dominant", or most abundant taxa in a sample, DTMM more effectively incorporates less abundant taxa. Whether obtained through DMM or DTMM methods, final clusters are considered bacterial communities that can be tested for associations with phenotypes.

Testing for Associations Between Bacterial Communities and MUI Versus Control Phenotype

Similar to the original analysis, in this updated analysis we assessed for associations between bacterial communities and clinical phenotype (MUI vs. control) using multivariable generalized linear models with a logit link. While our primary analysis focused on bacterial communities generated from DMM methods, we also assessed models that incorporated bacterial communities generated through DTMM clustering methods to assess how clustering methods might influence results. Both original and updated analyses incorporated several covariates though some of these were chosen differently, as detailed in **Supplemental Table 1**. In both original and updated analyses, bacterial community types were included in models with the following covariates: age, ethnicity, BMI, and smoking status. However, in the original analysis age was strongly associated with bacterial communities. Thus, investigators performed *post hoc* sub-analyses in participants < 51 years and those with ages 51 and older, with the age of 51 chosen since it is the median age of menopause in the United States (Komesu et al., 2018). For the updated analysis, we elected to include menopausal status into our model, but also needed to appropriately manage hormone therapy that occurs with menopause. To do this, we created a composite variable that incorporated menopausal and hormonal status as one of three categorical options: 1) pre-menopausal; 2) post-menopausal with any estrogen hormone use (topical, vaginal, transdermal, oral); and 3) post-menopausal without hormone use. In addition, we included vaginal pH in updated models. Finally, we added two covariates into updated models because of their relevance to the microbiome - history of recurrent UTI, and number of days from the most recent catheterization (calculated based on last prior recorded catheterized urine sample or urodynamic assessment). These variables have been proposed as "desired" within recently published standards for urinary microbiome research (Brubaker et al., 2021). Both the original and updated analyses considered clinical site where samples were acquired, though site was managed differently in original versus updated models (see **Supplemental Table 1** & **Supplemental Figure 1**). Multivariable modeling was performed in R using stats v4.0.5.

[5]https://github.com/MaStatLab/DTMM

RESULTS

Unprocessed sequencing data from 207 samples (123 MUI and 84 controls) that were sequenced using 300bp paired-end reads from V1-V3 and V4-V6 variable regions were reprocessed for this analysis. This resulted in taxonomic data in 173 samples for the V1-V3 region and 194 samples for the V4-V6 region. When attempting to merge forward and reverse reads from the V1-V3 region, there was substantial data loss such that reads from approximately 25% of the samples would have been removed from the dataset. Similarly, sequencing reads from the V4-V6 region, which provides a longer amplicon, were unable to be merged because of lack of enough overlapping sequence (see **Figure 1**). Given these constraints in data from both amplicons, we used forward reads only for subsequent analyses. It is unclear if the reads were merged or unmerged in the original analysis which used the Illumina BaseSpace 16S Metagenomics App for sequence processing. Median classified reads (i.e. recovered taxa) from the ASV table are summarized in **Table 1** and compared to those from the original analysis. For both amplicons in this dataset, we observed a median of 301 base pairs (bp) in sequencing read length. Given that we used forward reads only, we assessed the variable regions that would have been spanned with the stated primers and ~301 bp of sequencing (see **Figure 1**). As such, the V1-V3 forward read covers all of V1 and most of the V2 region. The V4-V6 forward read mainly comprises the V4 region, as it is not long enough to span V5 and V6 regions. For improved transparency and accuracy, in the remainder of this manuscript we will refer to the data as those arising from the V1-V2 regions and V4 regions, respectively.

Comparisons of Recovered Taxa in Original Versus Updated Analysis

When comparing the originally processed OTU table and updated ASV table from data generated by the V4 amplicon, 329 genera were only identified with original processing, 426 were only identified with updated processing, and 289 were overlapping and identified with both (**Figure 3A**). Though there were many non-overlapping genera, these were represented in the small proportion of the low abundance

TABLE 1 | Sequencing data & Recovered taxa.

	Median (range) classified reads	# Phyla	# Classes	# Orders	# Families	# Genera
V1-V2*	24,862 (1,021 – 670,442)	29	63	143	220	545
V4*	29,105 (5,029 – 187,593)	27	73	182	256	721
Original analysis V4-V6 (5)^	55,163 (2,835 – 205,548)	28	60	82	191	581

**Due to technical issues when merging forward and reverse reads while using a pipeline that generates amplicon sequence variants (ASVs), only forward reads were used in updated bioinformatic processing.*

^The original publication describes the sequencing read depth prior to bioinformatic processing. The number of classified reads are not listed, but were extracted from OTU tables provided from the PFDN for this updated analysis.

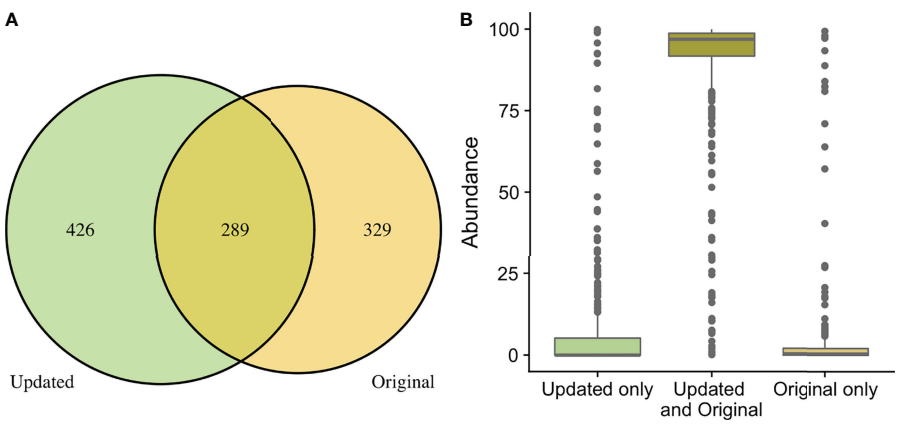

FIGURE 3 | Comparison of taxa recovered through original and updated processing. OTU tables from the original study were obtained and compared to data generated from ASV tables after repeat processing. **(A)** depicts the number of individual genera that were identified after the original and updated processing pipelines. There were 329 and 432 unique genera identified with the original and updated pipelines, respectively. A total of 289 genera were identified with both processing pipelines. **(B)** shows the relative sequence abundances of those identified with only original or updated processing, as well as those identified with both pipelines. As depicted, the unique genera that are only identified in the original or updated pipelines tend to be low abundance sequences, while the large majority of the highly abundant sequences were identified through both processing pipelines.

sequences from all samples (**Figure 3B**). The 329 genera that were uniquely identified with original processing are included in **Supplemental Table 2** and had counts ranging from 2-211. The 426 genera that were uniquely identified with updated processing are included in **Supplemental Table 3** and had counts ranging from 1-178. Of these, a total of 179 genera had a mean relative abundance <0.0005 and 66 genera had a mean relative abundance <0.0001, which were the filtering thresholds used in this analysis. Non-overlapping genera from original and updated processing with mean relative abundances >0.2 are displayed in **Table 2**.

Comparisons of Recovered Taxa by Sequencing Amplicons

In the updated analysis, a total of 164/207 (79%) of samples had paired classified taxa from V1-V2 and V4 regions (generated after sequencing V1-V3 and V4-V6 amplicons, respectively). Of these, 113 genera were only represented in the V1-V2 dataset, 279 were only represented in the V4 dataset, and 420 were overlapping and represented in both the V1-V2 and V4 datasets (**Figure 4A**). Like patterns detected when comparing OTU and ASV tables, the most abundant genera were overlapping and identified in both amplicons, while non-overlapping genera were identified in low abundance sequences (**Figure 4B**). Of the taxa that were represented in both V1-V2 and V4 regions, the median abundance was 99.3%. Of the taxa that were only represented in V1-V2, the median abundance was 0.35%; of the taxa that were only represented in V4, the median abundance was 0.88%. Taxa recovered per sample from V1-V2 and V4 regions are shown in **Figure 5**. Paired abundances from V1-V2 and V4 regions from the most highly abundant genera are summarized in **Figure 6**, with the remaining genera summarized in **Supplemental Figure 2**. A higher relative abundance of *Lactobacillus* was

TABLE 2 | Unique taxa with highest mean abundances from original and updated processing.

	Genus	Count	Maximum Relative Abundance	Minimum Relative Abundance	Mean Relative Abundance
Original Processing	*Serratia*	200	95.8583359	0.00107388	2.67694661
	Escherichia	120	77.6694728	0.00072271	2.13110697
	Clostridium	210	82.1156486	0.00230984	0.6235074
	Enterobacter	108	43.4458227	0.00072271	0.23072134
Updated Processing	*Escherichia/Shigella**	85	99.8775177	0.01175254	4.91545093
	Cutibacterium	178	58.8692498	0.01118443	3.94134097
	Actinotignum	53	64.7708383	0.00987882	0.62764323
	Clostridium_sensu_stricto^	44	88.5600496	0.00498915	0.52225445
	Proteus	9	71.5686275	0.0122444	0.37338724
	Ezakiella	72	13.1103903	0.003139	0.22165684
	Methylophilus	67	4.84244259	0.00356837	0.21395235

Only taxa with mean relative abundances >0.2 listed here. For full lists of unique taxa from original and updated analyses, refer to ***Supplemental Tables 2, 3****, respectively.*
**The SILVA database classifies as Escherichia/Shigella while other databases (e.g., Greengenes in the original analysis) classify as Escherichia. Thus, this classification appears unique within original and updated datasets but could refer to similar genera.*
^Classified as Clostridium_sensu_stricto in updated analysis using the SILVA database. It is unclear if this is a subset of Clostridium, or if this name in SILVA refers to the genus Clostridium from the Greengenes database.

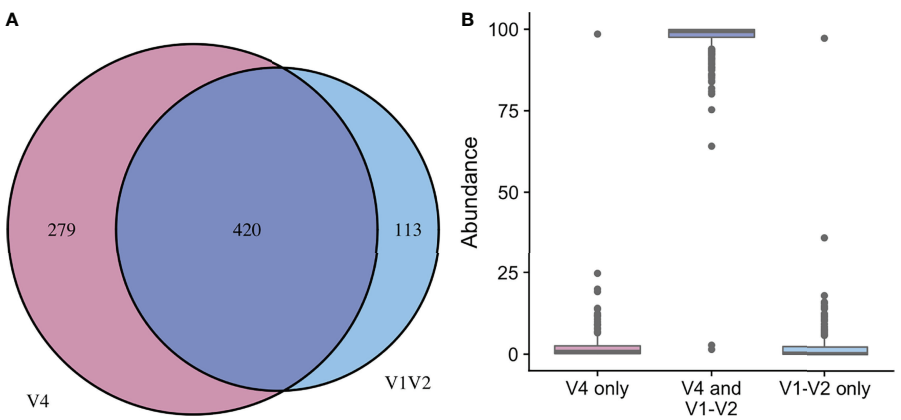

FIGURE 4 | Comparison of taxa recovered from different sequencing amplicons. For samples with classified taxa from both V1-V2 and V4 regions (n=164), paired ASV tables were compared. **(A)** shows that 113 unique genera were identified with the V1-V2 region, 279 unique genera were identified with the V4 region, while 420 genera were shared and identified with both regions. **(B)** shows the relative sequence abundances of those identified with each region, as well as those that were identified with both regions. The unique genera that are only identified with one region are extremely low abundance sequences with an occasional outlier, while the large majority of the highly abundant sequences were identified in both regions (i.e., V1-V2 and V4 regions).

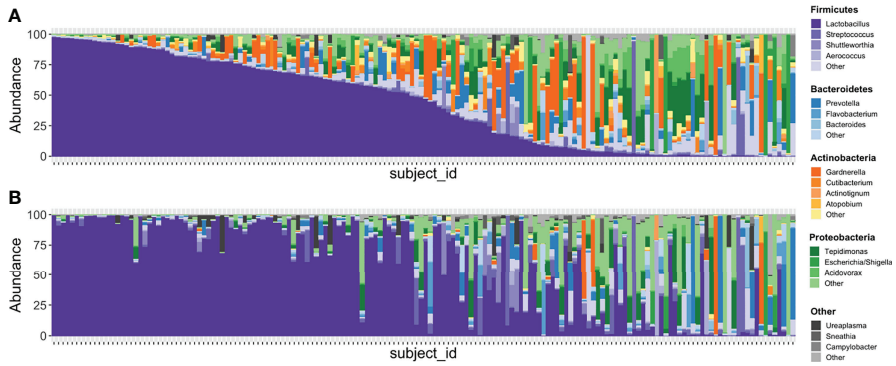

FIGURE 5 | Stacked bar plots illustrating relative abundances of taxa in 167 samples with paired V1-V2 and V4 data. **(A)** depicts the taxa recovered with the V4 region while **(B)** depicts the taxa recovered with the V1-V2 region. Each vertical bar depicts an individual sample with plots aligned to compare recovery of data from the same sample in each amplicon. Phyla are assigned distinct colors (e.g., Firmicutes = purple, Bacteroidetes = blue, Actinobacteria = orange, Proteobacteria = green) with individual genera shaded differently. The most intense color shade within each phylum refers to the most abundant genus identified. Though many genera are recovered in similar abundances between both amplicons, *Gardnerella* is one that is noticeably different, with substantially more identified in sequencing data generated from the V4 region.

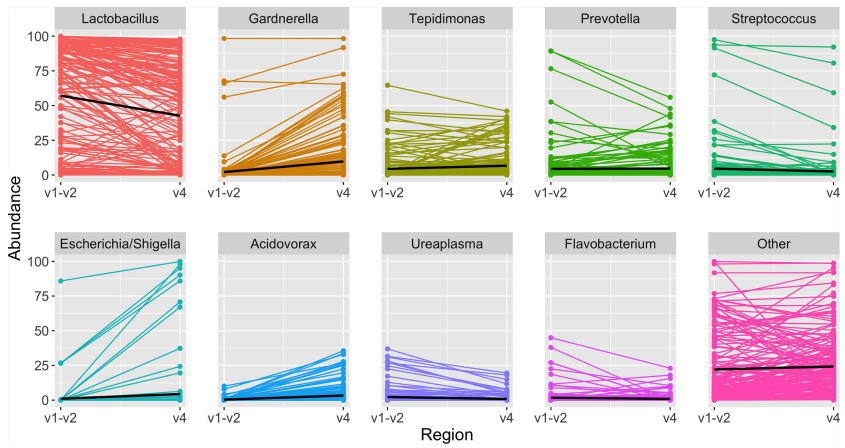

FIGURE 6 | Highest abundance genera among paired samples. Each panel depicts the relative abundance of one genus. On the left is the relative abundance from the V1-V2 amplicon, connected by a line to the right, which shows the relative abundance in the same sample when identified from the V4 amplicon. In each panel the black line summarizes the median abundances across all paired samples. When comparing results from the same sample sequenced with two different amplicons, *Lactobacillus* was identified in slightly higher abundance with the V1-V2 amplicon, while other genera including *Gardnerella, Tepidomonas, Escherichia/Shigella*, and *Acidovorax* were identified in slightly higher abundance with the V4 amplicon.

identified with the V1-V2 region, while slightly higher relative abundances of *Gardnerella, Tepidomonas, Escherichia/Shigella*, and *Acidovorax* were identified with the V4 region, with subtle differences in other genera. Without further testing and validation, it is unknown which of these two regions more accurately reflect true bacterial presence in the urinary bladder. However, multiple factors led us to infer that the V4 data might be more reliable in this dataset. First, in earlier stages of processing, it was noted that the V1-V2 region contained many sequences mapping to non-bacterial taxa (e.g., archaea, eukaryote, or not assigned) when compared to the SILVA reference database while the V4 region mapped mainly to bacterial taxa, as expected. Secondly, the V1-V2 region recovered *Gardnerella* in a sparser manner than the V4 region did. *Gardnerella* are biologically expected when reviewing prior urinary and vaginal microbiome data. Based on these considerations, we considered the V4 region data to be more reliable, and these data were selected for statistical analyses. This mirrors the original analysis, in which the authors elected to focus only on V4-V6 region sequencing results in their publication (Komesu et al., 2018).

Testing for Associations Between Individual Taxa and Clinical Phenotypes

In BGCR analysis, we did not identify differences in microbial composition between MUI and control participants after

adjusting for clinical covariates (PJAP = 0.337, indicating only a 33.7% probability of differences in the individual taxa).

Clustering Into Bacterial Communities

As was done in the original analysis, we created a sub-structure within the microbial data by clustering samples into bacterial communities. We first repeated the original strategy using DMM modeling with the reprocessed data. In DMM modeling, the number of final clusters are pre-specified. Since the original analysis selected 6 clusters, we chose the same number for the updated analyses. **Figure 7** shows the 6 DMM clusters (i.e., bacterial communities) that we identified with filtered reprocessed data grouped by MUI and control phenotypes. We also clustered filtered reprocessed data using DTMM modeling where the number of clusters are mathematically chosen based on the data. Using the DTMM approach, there were only 3 clusters when filtering at 0.0005, though a 4th cluster appeared when using a less stringent filtering threshold of 0.0001 (**Supplemental Figure 3**). This illustrates how multiple analysis steps, including the filtering strategy and clustering method could influence overall results, and thus should be carefully selected to best illustrate data without over-emphasizing "noise" within the dataset.

Testing for Associations Between Bacterial Communities and MUI Versus Control Phenotype

Multivariable models were used to determine whether bacterial communities were associated with MUI versus control status, while controlling for other relevant covariates. We first created models that incorporated the clinical site. While some of the sites were significantly associated with outcomes, associations with clinical site were not stable among different models (see **Supplemental Information**). To avoid overfitting models, clinical site was removed in final models, which incorporated bacterial communities and the following covariates: age, smoking status, ethnicity, BMI, composite menopausal/hormonal status, vaginal pH, history of recurrent UTI, and number days from the most recent catheterization.

In our updated analysis using re-processed and filtered data as well as bacterial communities generated from DMM clustering, Cluster 5 was associated with MUI (p=0.03) with a trend towards

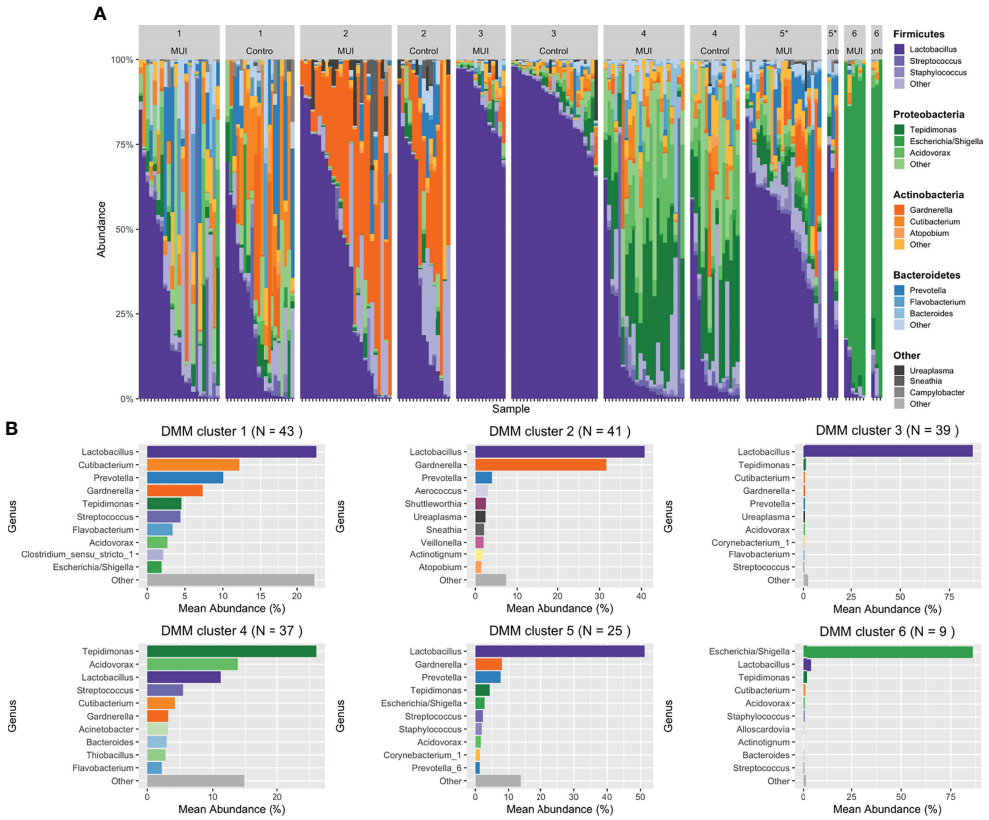

FIGURE 7 | Stacked bar plots illustrating results from updated analysis when combining filtered ASVs from individual samples into bacterial communities using DMM clustering. For DMM clustering a total of 6 clusters were chosen *a priori*. Clusters are numbered with each cluster further organized by those samples originating from women with MUI versus control. **(A)** shows DMM clustering results with cluster membership that is significantly different between MUI and controls identified with an asterisk(*). **(B)** shows more detail about the relative abundances of various genera that contribute to each cluster.

cluster 3 being associated with controls (p=0.08). Cluster 5 refers to one with moderate *Lactobacilli* (mean relative abundance of ~50%), followed by almost equal *Gardernella* and *Prevotella* (mean relative abundances of 8.5-9%) with several other low abundance genera (see **Figure 7B**). Cluster 3 has much higher abundance of *Lactobacilli* (mean relative abundance almost 90%) with very small components of others (see **Figure 7B**). The covariates BMI and Latina ethnicity remained significantly associated with MUI, even when controlling for other variables, including bacterial community types.

The updated analyses that best approximates what was performed in the original analyses using unfiltered data is one where the least restrictive filtering threshold of 0.0001 is applied. Using this filtering threshold, DMM clustering was repeated giving 6 new bacterial communities, as depicted in **Supplemental Figure 4**. **Table 3** summarizes the results when the same model and clustering technique is used on data that are filtered differently. With a less restrictive threshold, Cluster 2 ($p < 0.05$) and Cluster 6 ($p = 0.01$), the latter with a composition similar to Cluster 5 above, were significantly associated with MUI while controlling for other covariates, including those that were also significantly associated with the MUI outcome, such as BMI ($p < 0.01$) and Latina ethnicity (p=0.02). In review of the actual taxa within clusters, it appears that the reference group in this model (Cluster 1) was characterized by very high abundances of *Lactobacilli* (see **Supplemental Figure 5**) and was the only group with a higher number of controls compared to MUI, despite the fact that control samples were under-represented in the overall dataset (~40% of overall samples).

A general inference from models incorporating bacterial communities is that communities with high proportions of *Lactobacilli* are associated with control status and communities with lower *Lactobacilli* and higher relative proportions of a combination of *Gardnerella and Prevotella* are associated with MUI status. Contrary to the original analysis, we did not perform subanalyses of participants age < 51 years and those 51 or older, but rather included the composite menopausal/hormonal status variable when modeling data. Our model also included history of recurrent UTIs, vaginal pH, and number of days since prior catheterization, since these are covariates that could contribute to further variability in urinary microbiome datasets.

We also performed multiple sensitivity analyses where we used the same modeling approach but with filtered data clustered with DTMM methods. DTMM generated fewer clusters (3 total) and we did not find signification associations between bacterial clusters generated with DTMM and the clinical phenotypes of MUI and control status. In these models, BMI and Latina ethnicity still remained significantly associated with MUI (p=0.007, p=0.004, respectively). Results are displayed in **Supplemental Figure 3**.

DISCUSSION

We re-analyzed previously generated sequencing data using updated bioinformatic techniques and refined the statistical analyses. This updated analysis offered several interesting nuances that enhance clinical inferences regarding the relationship between the urinary microbiome and MUI. In the original publication, researchers did not find differences in bacterial community types among women with MUI and controls, though a *post hoc* analysis found some associations between bacterial communities and MUI exclusively in women < 51 years of age. With our updated approach to the data, we first examined whether individual taxa might be drivers of differences between MUI and control phenotypes. Using BGCR analysis, we did not find this to be the case, as there was a low probability of differences in microbial composition between MUI and controls. However, similar to the original analysis, we assessed for how substructures within the microbial data (e.g., bacterial communities) might be associated with MUI versus control phenotypes. We were indeed able to confirm that associations between bacterial communities and MUI exist. However, after incorporating a variable that accounts for menopausal/hormone

TABLE 3 | Updated analysis multivariable model testing for associations between MUI versus control.

Variable	Less restrictive filtering threshold (0.0001)p value	Conservative filtering threshold (0.0005)p value
Microbial community by DMM clustering		
Cluster 1	(reference)	
Cluster 2	0.045*	0.605
Cluster 3	0.674	0.079
Cluster 4	0.091	0.415
Cluster 5	0.400	0.030*
Cluster 6	0.010*	0.883
Age (years)	0.107	0.177
Latina Ethnicity	0.017*	0.023*
Body Mass Index (kg/m^2)	0.002*	0.015*
Smoking Status	0.272	0.169
Vaginal pH	0.304	0.278
Menopause/Hormone Status^	0.403	0.626
Recurrent UTI	0.995	0.996
# days since prior catheterization	0.992	0.992

DMM (Dirichlet multinomial mixture); UTI (urinary tract infection)
**Significant association with MUI (mixed urinary incontinence)*
^Composite variable of menopausal status and presence of hormone

status in our model, we no longer found that associations differ by age. Even when sequencing data were filtered differently, associations between bacterial communities and MUI status remained robust with slightly different actual clusters (i.e., community members). This leads us to conclude that there is not likely to be one bacterial genus alone, but rather a difference in communities of bacteria, and perhaps how they interact, that is associated with mixed urinary incontinence phenotypes.

With this updated analysis we found that an updated bioinformatic processing pipeline recovers many different taxa compared to prior bioinformatic techniques. However, most of these differences exist in low abundance taxa that occupy a small proportion of the overall microbiome. We also confirmed that in urine, similar to other sample types, the region of the 16S rRNA gene that is chosen for sequencing can impact downstream results. For the most common (highest abundance) taxa, information will be recovered regardless of the bioinformatic strategy. However, less abundant taxa may have different biases based on the bioinformatics and sequencing amplicon chosen. For less abundant taxa, results may require additional validation and should be considered carefully when attempting to make inferences.

Strengths of our approach include the application of techniques that improve precision when analyzing low biomass samples. The bioinformatic processing pipeline applied in this study (i.e., DADA2) corrects for sequencing errors and chimeric sequences to improve accuracy. For updated processing we also used a different reference database (i.e., SILVA), since Greengenes, a database used in many prior urinary microbiome studies, has since been shown to have poor representation of bladder bacteria (Hoffman et al., 2021) and has not been updated since 2013. However, our bioinformatic approach is limited as specific expertise (e.g., knowledge of how to use R and other microbiome processing software like QIIME2[6]) may be required compared to prior "plug and play" approaches like the Illumina BaseSpace software. With enhancements in precision, we also encountered more data loss, as some samples did not have high enough quality sequencing information to provide taxonomic data. While we acknowledge that this may decrease the sample size, it may inherently be more scientifically rigorous to remove lower quality sequencing information. Despite technical differences in how sequencing data are handled, our updated processing identified a similar number of phyla and classes compared to the originally processed data (**Table 1**), with significantly more orders, families, and genera compared what was originally reported.

Another strength to our approach is that we tested multiple aspects of statistical analyses, including various filtering and clustering approaches, prior to arriving at our conclusions. Results from these sensitivity analyses offer insights to the urobiome community, as the filtering thresholds and clustering methodology chosen for a study may affect interpretation of overall results. Generally, researchers need to decide if they want to filter at a lower threshold, thereby keeping more sequencing data. With this approach, there is a risk of over-interpreting data in low biomass samples based on possible contaminants or low abundance sequence information. The other alternative is to filter at a higher threshold, which removes more data, but could result in missing an important association because clusters are less refined. This concept is illustrated in our study when evaluating multivariable models using DMM clustering to create microbial communities. In models with a less restrictive filtering threshold, there were associations that appeared statistically meaningful. When using the same clustering methodology with a more conservative filtering threshold, there are still statistically significant associations, but the clusters and downstream inferences are slightly different. Ultimately, it is only with repeated experiments and ongoing validation that we will expect to understand which approach best approximates the truth. However, it is important for investigators to understand how these choices that are made during statistical analyses may affect downstream results.

Existing groups are applying published techniques extrapolated from linear mathematical modeling to analyze microbial datasets. However, many of these techniques contain underlying assumptions of normally distributed data. High dimensional microbial datasets that are used in community-based analyses fail to meet these underlying assumptions, and thus additional techniques are being evaluated and developed. We had hypothesized that tree-based clustering approaches (e.g., DTMM) may be able to better resolve true signal from noise within a dataset. Compared to DMM clustering, DTMM puts more emphasis on lower abundance taxa when clustering. Incorporating a nonparametric mixture as in the available implementation of DTMM also avoids the often-difficult task of pre-specifying the number of clusters. While it was not the case that DTMM clustering was able to better resolve signal from noise in this analysis, it is still possible that other models more akin to machine learning may be useful in the future. For urinary microbiome data it is also not clear if the ratio of high to low abundance taxa (e.g., ratio of *Lactobacilli* compared to other Gram negative & anaerobic bacteria) is more biologically important or if individual low abundance taxa may be important. If the ratio of high abundance bacteria compared to all other bacteria is actually the most biologically important factor, then a clustering method such as DMM that emphasize the highest abundance taxa may actually be preferred.

Compared with the original analysis, we came to slightly different conclusions when evaluating results from our final multivariable models. While we agreed that there were associations between microbial communities and MUI, the context of these associations was different in our updated analysis. Specifically, in the original analysis, 17% of women reported their menopausal status as unknown prompting investigators to dichotomize age based on the approximate age of menopause (51 years) and analyze data in those less than 51 and those older than 51 years. With this approach there were different findings in the two sub-populations (Komesu et al., 2018), which is somewhat difficult to interpret. Furthermore, hormone status (e.g., whether oral or topical/vaginal hormones were used) was not incorporated into multivariable analyses despite differences noted in MUI and control populations.

[6]https://qiime2.org/

Multiple investigators have demonstrated that menopause and hormonal status affect microbial compositions in the vagina (Brotman et al., 2014; Gliniewicz et al., 2019), and we are now learning that these variables are associated with differences in microbial compositions of the bladder as well (Thomas-White et al., 2020). As such, the original clinical data were reviewed to assess how these data were obtained. In this process, we discovered that menopausal information was obtained twice, with one group of questions having more reliable response options. Furthermore, two clinicians (NYS and LB) reviewed all age, menopause, and hormone usage information. Using a combination of these responses, we were able to reliably create a composite variable that incorporated menopausal & hormonal information in an accurate manner. In addition to this composite variable, additional variables that could also confound microbial information such as vaginal pH, history of recurrent UTI, and number of days from prior catheterization were also incorporated into multivariable models, while they were not previously. With this modeling strategy, we no longer see age as a separate independent factor affecting microbial community types. Regardless of the modeling strategy used, multiple covariates remained associated with the bladder outcome of MUI, highlighting the importance of incorporating covariates into analyses of microbial data.

A limitation in our updated analysis is that we had to rely on previously generated sequencing information and were not able to influence laboratory aspects of the study. For example, the choice of using the V4-V6 amplicon with very short overlapping sequences resulted in the inability to merge forward and reverse sequencing reads during bioinformatic processing to create final reads with longer length. While the V1-V3 region had more overlapping sequence between forward and reverse reads, there were still similar issues in attempting to merge reads that would have resulted in substantial data loss. It is not clear how these issues were managed in the original analysis when using the Illumina BaseSpace Metagenomics App, which is a "black box" bioinformatics approach. Ultimately, for the updated analysis we chose to use forward reads only. When comparing the taxa recovered, both based on numbers of taxa classified (**Table 1**), as well as comparisons of the classifications between original and updated analyses (**Figure 3**), we have inferred that our analysis of forward read only data very closely approximates the information provided in the original analysis, which was stated to use merged reads.

Another limitation is that any sequencing method that uses shorter lengths of DNA (e.g., what occurs with one or two variable regions of the 16S rRNA gene) could result in some difficulty classifying sequences at higher resolutions such as genus or species. Thus, newer techniques that incorporate highly accurate long-read sequencing methods (Callahan et al., 2019; Callahan et al., 2021) may be helpful to characterize the microbiome in a new niche. We were limited to using previously generated sequencing data and thus could not take such steps to enhance accuracy and resolution of the dataset, which might prove to be useful. Also related to the goal of enhancing accuracy, many studies will now incorporate a mock microbial community with serial dilutions to allow for the application of additional methods of removing contaminant ASVs during bioinformatic processing (Davis et al., 2018; Karstens et al., 2019). Since the sequencing data used in this study were developed prior to large scale incorporation of this approach, a mock microbial community was not used. Given that urine is a low biomass sample type that may be influenced by contaminants, future studies would likely benefit from the incorporation of current methods to remove contaminant ASVs during bioinformatic processing. This strategy may also facilitate the ability to use less stringent filtering thresholds since many contaminants will have already been removed. However, Cao *et al.* recently provided data that bioinformatic contaminant removal and filtering are complementary methods and should be employed together in highly rigorous studies (Cao et al., 2020).

With the continued evolution of computational techniques, we expect further improvements and guidelines for analyzing microbial datasets. With this updated analysis, we offer additional insights for investigators embarking on urinary microbiome analyses, and also enhanced clinical inferences regarding the relationship between the urinary microbiome and MUI. Specific considerations should be given to the amplicon (i.e., region of 16S rRNA gene) chosen, the bioinformatic processing pipeline, and the reference database that is used to ensure that updated resources containing adequate representation of urinary microbiota are used. Though default filtering thresholds and clustering methodologies exist, these parameters may need to be optimized based on the questions that are being posed in a microbial dataset. Finally, regardless of how analyses are conducted, multivariable analyses that incorporate potentially confounding clinical variables remain extremely important in analyses of microbial datasets.

DATA AVAILABILITY STATEMENT

Unprocessed sequencing files are publicly shared on the Sequence Read Archive (SRA), Bioproject ID 703967, Accession #: PRJNA703967. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

This re-analysis of sequencing data from human participants was reviewed and approved by the Duke University Institutional Review Board (Pro #00102155). The patients/participants provided their written informed consent at the time of inclusion in the original HMS-ESTEEM study.

AUTHOR CONTRIBUTIONS

All authors have made substantial contributions as follows: conception or design of the work; or the acquisition, analysis or interpretation of data for the work (NS, LM, LB, JM, CH, ED, ZW, LK) drafting the work or revising it critically for important intellectual content (NS, LM, LB, LK) provide approval for

publication of the content (NS, LM, LB, JM, CH, ED, ZW, LK) agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved (NS).

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.789439/full#supplementary-material

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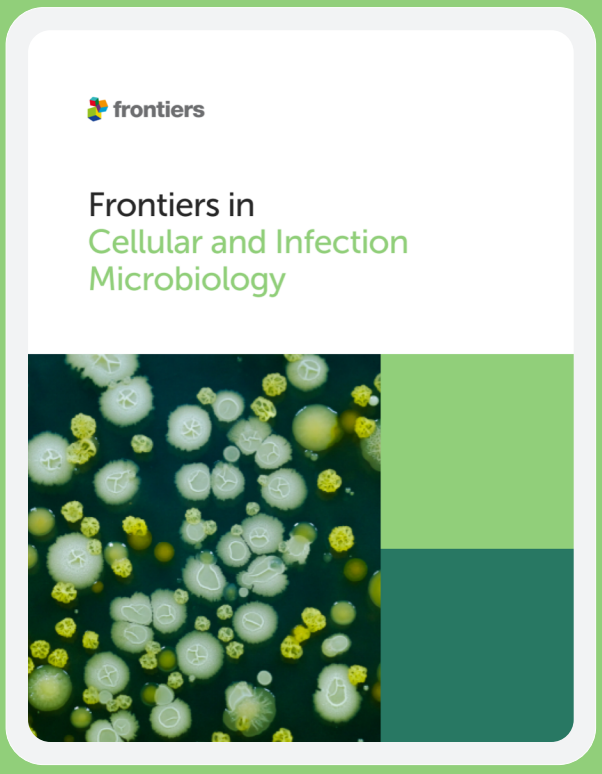

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