

## A Prospective Study of the Urinary and Gastrointestinal Microbiome in Prepubertal Males



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<b>OBJECTIVE</b>	To determine if urinary microbial communities similar to those described in adults exist in children and to profile the urinary and gastrointestinal microbiome in children presenting to urology for both routine and complex urologic procedures.
<b>METHODS</b>	Prepubertal boys (n = 20, ages 3 months-8 years; median age 15 months) who required elective urologic procedures were eligible. Urine samples were collected via sterile catheterization and fecal samples were obtained by rectal swabs. DNA was extracted from urine pellet and fecal samples and subjected to bacterial profiling via 16S rDNA Illumina sequencing and 16S rDNA quantitative polymerase chain reaction. We assessed within and between sample diversity and differential species abundance between samples.
<b>RESULTS</b>	Urine samples had low bacterial biomass that reflected the presence of bacterial populations. The most abundant genera detected in urine samples are not common to skin microbiota and several of the genera have been previously identified in the urinary microbiome of adults. We report presumably atypical compositional differences in both the urinary and gastrointestinal microbiome in children with prior antibiotic exposure and highlight an important case of a child who had undergone lifelong antibiotic treatment as prophylaxis for congenital abnormalities.
<b>CONCLUSION</b>	This study provides one of the first characterizations of the urinary microbiome in prepubertal males. Defining the baseline healthy microbiome in children may lay the foundation for understanding the long-term impact of factors such as antibiotic use in the development of a healthy microbiome as well as the development of future urologic and gastrointestinal diseases. UROLOGY 131: 204–210, 2019. © 2019 Elsevier Inc.

Recent years have brought a paradigm shift in our understanding of the complexity of microbial communities associated with the human body. The microbiome is a collection of micro-organisms (eg, the human-associated microbiota) that commensally exist in different regions of the body. Microbiota assist in maintaining healthy states in the human body including homeostasis and immune defense.<sup>1</sup> However, alterations to the microbial community structure are also implicated

in disease. Dysbiosis is a term used to describe changes in microbial communities that are associated with disease states. For instance, dysbiosis of the gastrointestinal (GI) tract has been associated with chronic inflammation, diabetes, obesity, and inflammatory bowel disease.<sup>2,3</sup>

In addition to known sites of microbial presence, such as the GI tract, vagina, and skin, studies have now shown an unexpected presence of microbial populations in areas of the human body once thought to be devoid of microbes. Notably, the urinary tract has long been thought to only be associated with microbes in the presence of disease. The long held clinical dogma that urine is “sterile” has been challenged by recent evidence. Careful studies demonstrate that bacteria profiled from urine samples both originated from the urinary tract and represent live bacteria.<sup>4,5</sup> Urine contains bacterial cells that are representative of a distinct flora in the urinary tract, including the urethra and the bladder (reviewed in<sup>6</sup>).

The urinary microbiome has been described in adults, and dysbiosis of the urinary microbiota has been associated with several urologic diseases including incontinence, interstitial cystitis, and sexually transmitted infection.<sup>6,7</sup> In

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addition, chronic inflammation caused by dysbiosis of the microbial communities in the urinary tract may be associated with the development of genitourinary cancers.<sup>8-10</sup> Although microbial communities of multiple sites of the body in children, including the GI tract, airway, and skin, have been previously studied,<sup>11-13</sup> there have been minimal reports to date that have aimed to characterize microbial communities in the urinary tract of children. One prior study characterized the microbiome of the mucosa of the native urinary bladder vs ileal segments used in bladder augmentation.<sup>14</sup> Therefore, it is currently unknown if there is a urinary microbiome in children, and if the microbial communities of the urinary tract in children differ from that which has been reported in adults. If the urinary tract is colonized in childhood, there may be implications for how alterations to the urinary microbiome may lead to development of pediatric urologic diseases (such as recurrent urinary tract infections) or influence urologic disease processes that appear in adulthood.<sup>15</sup>

In the present study, we hypothesized that the urinary microbiota likely begins colonizing the urinary tract in early childhood. Additionally, external factors, such as antibiotic exposure, may lead to alterations of the microbial community structure in the urinary tract. Thus, we aimed to (1) characterize the urinary microbiota of pediatric male patients and (2) compare the urinary and GI microbiome in children with and without prior antibiotic exposure. Our study focused on the male urinary microbiota given our aim to compare our findings to previous characterization of the urinary microbiota in adult men.<sup>10</sup>

## MATERIALS AND METHODS

**Sample Collection and Patient Population.** All samples were collected by a single surgeon under a Johns Hopkins Medicine institutional review board approved protocol with written informed consent obtained for all procedures performed as part of sample collection. Urine samples were collected from male pediatric patients prior to their elective urologic procedures while under anesthesia. A transurethral catheterization was performed once the genitalia was prepped and draped per JHH operating room protocol and prior to routine single dose of weight-based prophylactic antibiotics (cefazolin or clindamycin). Fecal samples were obtained at the same encounter via rectal swabs (VWR, Radnor, PA, Cat. no. 552C.BX). Details of the patients and sample characteristics are given in [Table 1](#). Samples were obtained from 20 male pediatric patients (ages 3 months-8 years; median age 15 months). Ten of the patients had prior antibiotic exposure and 10 did not. We obtained urine samples from 5 of the patients and fecal samples from all 10 of the patients in each group

(antibiotic exposure vs no antibiotic exposure). The antibiotic exposure group included patients who had a history of antibiotic treatment/prophylaxis prior to enrollment in the study ([Supplemental Table S1](#)).

**Sample Processing and DNA Isolation.** The 15 mL urine samples were handled using sterile technique and were pelleted by centrifugation at 1000 × gravity for 10 minutes within less than 4 hours of collection. Urine pellets or fecal swab contents were re-suspended in a total volume of 500 μL 1 × phosphate buffered saline and DNA was extracted as previously described.<sup>10</sup> Two negative control blank (500 μL 1 × phosphate buffered saline as starting material) DNA extractions were performed to control for contamination from DNA extraction through the full amplification and sequencing pipeline.

**16S rDNA Gene Library Preparation and Sequencing.** The V6 hypervariable region of the 16S rRNA gene was amplified using a 2-step polymerase chain reaction (PCR) strategy as previously described.<sup>10</sup> PCR products were visualized on agarose gel, gel extracted, and pooled before submission to the Sidney Kimmel Comprehensive Cancer Center Next Generation Sequencing Core at The Johns Hopkins Hospital for next generation sequencing on a HiSeq instrument. The 16S rDNA gene sequences have been submitted to the Short Read Archive (SRA) under BioProject PRJNA552861.

**Universal 16S rDNA Real-Time PCR.** We used a universal 16S rDNA quantitative PCR assay using the first round V6 primer set to quantify bacterial load in each of the urine pellet samples as previously described.<sup>10</sup> The number of 16S rDNA copies was quantified in relation to a standard curve of known copies of *Escherichia coli* DNA.

**Data Analysis.** Sequencing produced between ~4-30-million paired-end reads per sample which were analyzed to: (1) determine bacterial profiles of each sample; (2) calculate intra- and intergroup diversity; and (3) identify differentially represented taxa, using the QIIME1<sup>16</sup> and MetaStats 2.0<sup>17</sup> software packages. Prior to analysis, reads were de-multiplexed and filtered to remove low-quality data, vector contaminants, adapters and chimeric reads, and the 2 reads in a pair were joined together. Sequences were filtered based on a minimum length of 50 base pairs.

Read assignment to operational taxonomic units (OTUs) was performed with QIIME1 using open clustering with the GreenGenes database clustered at 99% sequencing identity, and taxonomic frequency profiles were created reflecting the community's OTU composition at different phylogenetic levels. Rarefaction plots of within-group (alpha) diversity were computed for the PD\_whole\_tree, Chao1, Shannon, and observed\_otus metrics. `compare_alpha_diversity.py` script was used to compare the alpha diversity (for the PD\_whole\_tree metrics) between groups of samples.

Between group, beta diversity was calculated with the weighted and unweighted unifrac similarity measures. A max sample depth of 807,980 (urine data set) and 808,384 (fecal data set) was used for both alpha and beta diversity. Lastly, the

**Table 1.** Characteristics of male pediatric patients whose urine and fecal samples were obtained immediately prior to urologic procedures at The Johns Hopkins Hospital (abx = antibiotic)

	Urine Samples (n)	Fecal Samples (n)	Average Age (months)	Median Age (months)	Range (Months)
No abx exposure	5	10	24	15	6-72
Abx exposure	5	10	29	19	3-96
Total	10	20			

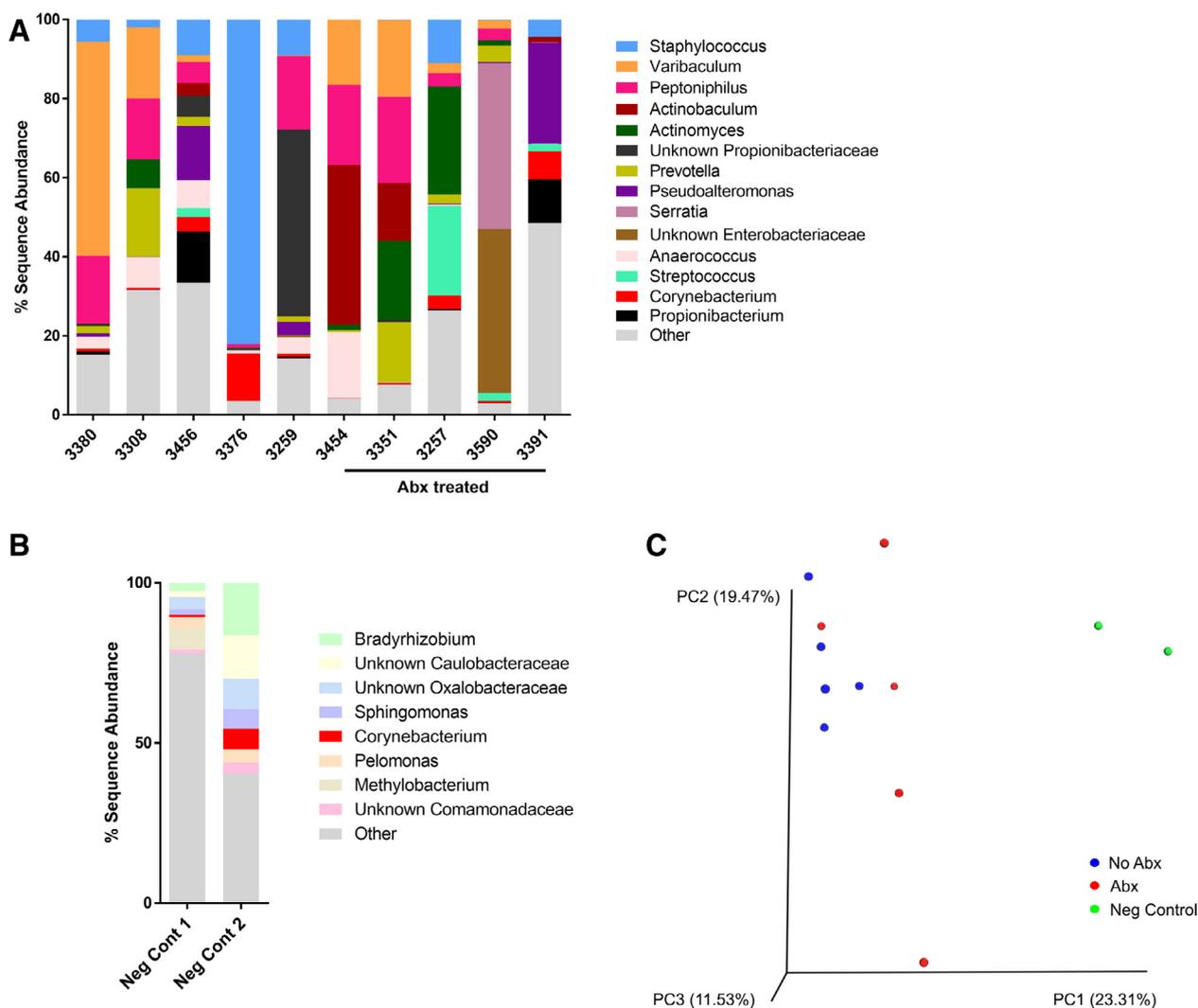
MetaStats 2.0 package was used to compare metagenomic profiles at each phylogenetic level, from kingdom to species, followed by Benjamini-Hochberg correction (q-values), to determine taxa that are statistically enriched or depleted in one condition compared with the other.

## RESULTS

**Characterization of the Pediatric Male Urinary Microbiome.** A total of 10 urine samples were analyzed from male children, including 5 male children with prior antibiotic exposure and 5 male children without prior antibiotic exposure. Notably, the estimated bacterial load in each urine sample was generally low, but higher than that of blank extraction negative controls (Supplemental Fig. S1). The median bacterial load was higher in urine from patients with prior antibiotic exposure than those without prior antibiotic exposure, although not statistically significant ( $P = .3$ , Mann-Whitney test).

The urine samples did not show a clear predominance of a particular bacterial genus across all patients. The majority of patients had representation from species of *Staphylococcus* and *Varibaculum* and to a lesser extent *Peptoniphilus* and *Actinobaculum* (Fig. 1A). The prevalent species detected in patient urine samples were not present in blank extraction negative controls (Fig. 1B). There were several differentially abundant genera between the antibiotic exposure group and the no antibiotic exposure group; however, these differences did not reach statistical significance when correcting for false discovery rate (Supplemental Table S2).

Within sample urinary microbial diversity (eg, alpha diversity) of patients with antibiotic exposure did not differ significantly from the urinary microbial diversity of patients without antibiotic exposure (difference in alpha diversity,  $P = .382$ ). This means that the total number of species (or species “richness”) and the presence of rare individual species were not different between the 2 groups. Principal component analysis (PCoA,



**Figure 1.** The urinary microbiome of pediatric male patients. (A) Urine samples ( $n = 10$ ) organized by bacterial genus. Relative composition of urinary microbial genera in each urine sample (Abx treated = antibiotic exposure group). Stacked bar plots demonstrate the 14 most abundant bacterial genera represented in each sample. (B) The most abundant genera found in blank extraction negative control samples do not overlap with the genera identified in urine samples. (C) PCoA analysis (Unweighted UniFrac) of urinary microbiota from patients with no prior antibiotic treatment (No Abx, blue circles), prior antibiotic treatment (Abx, red circles), or blank extraction negative controls (Neg Control, green circles). (Color version available online.)

a measure of relatedness between samples) demonstrated clear clustering of the urine microbial reads away from the blank extraction negative controls (Fig. 1C). This is important in establishing that the species identified in the urine samples likely were not the result of exogenously introduced contaminating bacterial DNA from either the DNA extraction or sequencing pipeline. Additionally, samples obtained from patients without prior antibiotic exposure clustered closer in proximity to each other than samples obtained from patients with prior antibiotic exposure. This suggests that there may be a greater dissimilarity between the bacterial compositions in urine samples of patients that have had antibiotic exposure compared to patients that have not had antibiotic exposure.

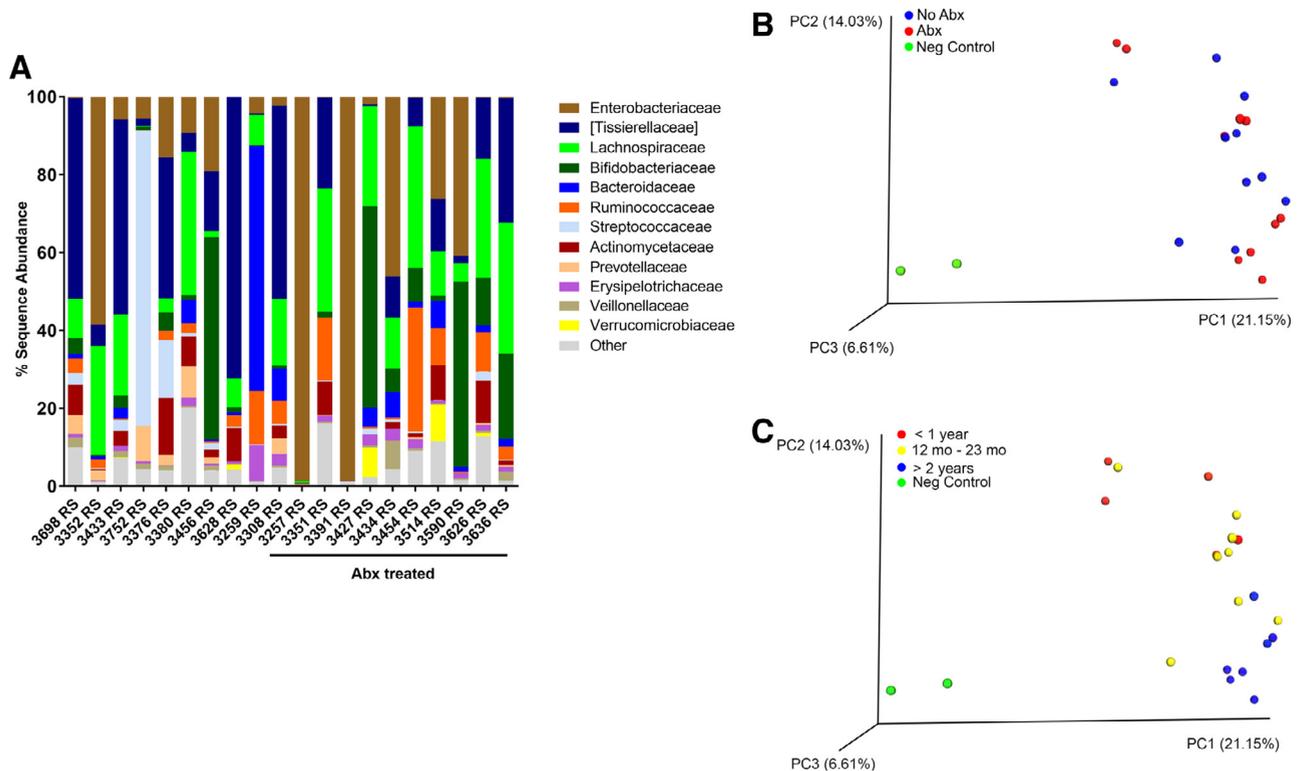
**Characterization of the Male Pediatric GI Microbiome in Relation to Antibiotic Exposure.** A total of 20 fecal samples were analyzed from male children, including 10 male children with prior exposure to antibiotics and 10 male children without prior exposure to antibiotics. The GI microbial sequences demonstrated a clear predominance of the phyla *Firmicutes* in most samples with *Bacteroidetes*, *Proteobacteria*, and *Actinobacteria* present in all samples as well (Supplemental Fig. S2). There were several significant differentially abundant genera among the GI microbiota in the antibiotic exposure vs no antibiotic groups, and select examples are shown in Supplemental Table S3. Notably, fecal samples 3257RS (from a 14-month-old) and 3391RS (from a 9-month-old) in the antibiotic exposure group were atypically dominated by the bacterial family *Enterobacteriaceae* (Fig. 2A). Overall, however, GI microbiota alpha diversity was not significantly

different between patients with vs without prior antibiotic exposure (difference in alpha diversity,  $P = .927$ ).

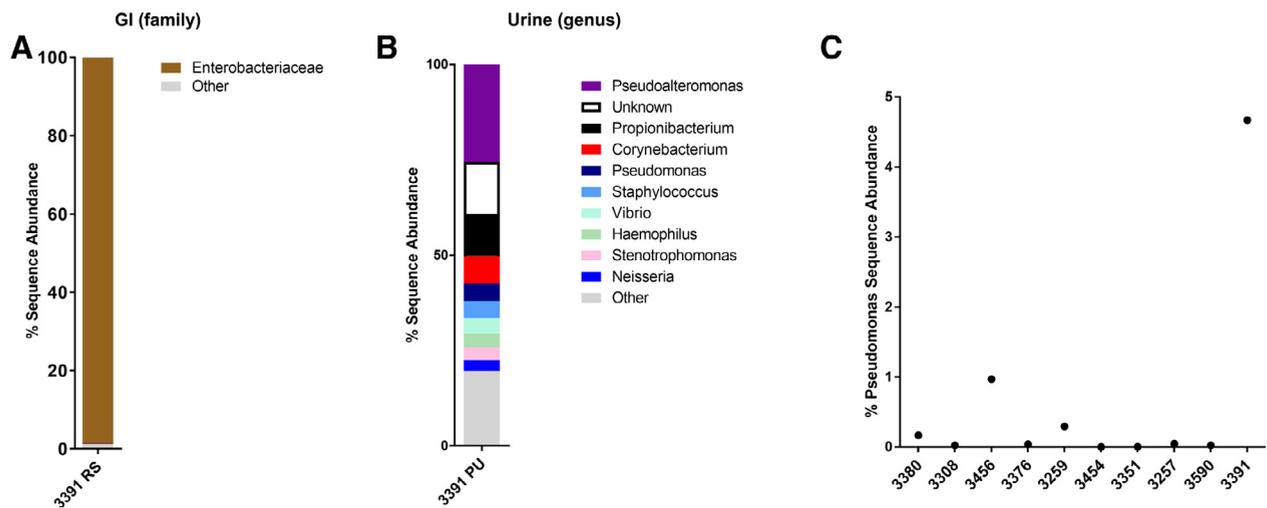
PCoA demonstrated no apparent clustering of the GI microbial reads between patients with and without prior antibiotic exposure, but clear clustering away from the negative control blank extractions (Fig. 2B). Of interest, we noted that the GI microbiota of children  $\geq 2$  years old clustered away from the rest of the samples (Fig. 2C).

**Clinical Implications of 16S rDNA Sequencing.** One case of interest to highlight from this study was a 9-month-old boy (patient number 3391) in the antibiotic exposure group. His clinical history was significant for a tethered cord, hydronephrosis of the left kidney, congenital imperforate anus, neurogenic bladder, and a septal heart defect. Prior to his ureteropelvic junction (UPJ) obstruction repair, he had already undergone multiple surgeries including septal heart defect repair, anorectoplasty, circumcision, and gastrostomy. Since birth, he had been on amoxicillin prophylaxis daily. After his UPJ obstruction repair, he did well postoperatively and was discharged on postoperative day 4. On postoperative day 6, he was readmitted with a fever and was found to have urosepsis with a positive urine culture for *Pseudomonas aeruginosa*.

Careful analysis of the 16S rDNA profiling of this patient's urine and GI sample sequences revealed interesting insights. His GI microbiota was dominated by *Enterobacteriaceae* (Fig. 3A). Over 50% of the sequencing reads obtained from this patient's fecal specimen were assigned to a single OTU identified as a species of *Enterobacter*. This OTU was found at well below 1% of



**Figure 2.** The gastrointestinal microbiome of pediatric male patients. (A) Fecal samples ( $n = 20$ ) organized by bacterial family. Relative composition of GI microbial family in each fecal sample (Abx treated = antibiotic exposure group). Stacked bar plots demonstrate the 12 most abundant bacterial families represented in each sample. (B) PCoA analysis (Unweighted UniFrac) of GI microbiota from patients with no prior antibiotic treatment (No Abx, blue circles), prior antibiotic treatment (Abx, red circles), or blank extraction negative controls (Neg Control, green circles). (C) PCoA analysis (Unweighted UniFrac) of patient samples organized by age of the patient. (Color version available online.)



**Figure 3.** Atypical GI and urinary microbiota in a 9-month-old patient treated with antibiotics from birth. (A) Fecal sample profile organized by bacterial family. (B) Urine sample profile organized by bacterial genus. (C) Percent sequence abundance of sequencing reads assigned to the genus *Pseudomonas* in each urine sample (n = 10). (Color version available online.)

the other patient samples (Supplemental Fig. S2). Upon further analysis of the urine sample profile, we also noted an increased percentage of reads assigned to the genus *Pseudomonas* compared to all other patient samples (Fig. 3B,C). Therefore, we speculate that 16S rDNA sequencing of the patient's urine sample revealed that his urinary tract had been atypically colonized by *Pseudomonas* prior to his clinical symptoms of urosepsis. Importantly, both *Enterobacter* spp. and *Pseudomonas aeruginosa* would be intrinsically resistant to amoxicillin.

## DISCUSSION

Our study demonstrates some of the first evidence of a urinary microbial community in the urinary tract of male children as young as 3 months old. We performed blank DNA extraction controls, which were sequenced and analyzed to confirm that the bacterial DNA sequences that we detected in urine samples were not simply reflective of exogenous contamination from the DNA extraction and sequencing reagents. Indeed, this can be a pervasive problem with the interpretation of low bacterial biomass samples,<sup>18</sup> and we carefully controlled for this issue. One other concern with the evaluation of the urinary microbiome is that the bacteria detected in the urine may only be representative of skin flora that were introduced into the sample during collection. We used urine collected via catheterization in the present study and whereas some genera detected in the urine are also commonly represented in skin flora (*Staphylococcus*, *Streptococcus*, *Propionibacterium*, and *Corynebacterium*), some of the most abundant genera detected in the urine samples (*Varibaculum*, *Peptoniphilus*, *Actinobaculum*, etc.) are not common to skin microbiota. Furthermore, many of the species identified in the urine samples are anaerobic microbes (for example, *Anaerococcus*, *Peptoniphilus*, and *Serratia*). Several of these genera have also been previously identified in the urinary microbiome of healthy individuals.<sup>19</sup>

Interestingly, urine bacterial load was low in most cases and slightly lower overall than the levels previously reported in adult men.<sup>10</sup> One hypothesis for the higher bacterial loads in some of the patients in the antibiotic exposure group vs those without antibiotic exposure (Supplemental Fig. S1) may include the opportunistic overgrowth of certain bacterial groups after antibiotic exposure, similar to what is seen in *Clostridium difficile* colitis.<sup>20</sup> In support of this, we noted that the urinary microbiota profile of the patients in the antibiotic treated group that had the highest bacterial load (patients 3454 and 3351, Supplemental Fig. S1) had an over-representation of certain bacterial taxonomic groups including *Actinobaculum* compared to all other samples. *Actinobaculum*, along with other genera overrepresented in the urine samples of the antibiotic treated group such as *Actinomyces* and *Serratia*, have been previously described as uropathogens.<sup>21-23</sup> One hypothesis includes the idea that antibiotic exposure may be allowing for potential pathogenic overgrowth in these patients. Overall, we noted that the urinary microbial communities that we profiled in children were different from those previously described in adults. In adult men, there is a predominance of *Staphylococcus* and *Corynebacterium*,<sup>10</sup> whereas the sequence reads from male children demonstrated a presence but not predominance of these genera of bacteria in most cases. We propose that the composition of the urinary microbiome in children may begin to develop early in life and evolve over time, becoming more stable in adulthood much like what has previously been reported for the GI microbiota.<sup>24,25</sup>

Additionally, we explored how prior antibiotic exposure may influence the GI microbiome in pediatric patients. The GI microbial profiles observed were largely in accordance with previously published studies of the GI microbiome in infancy and early childhood (reviewed

in<sup>26</sup>) with an early representation of *Enterobacteriaceae* and *Staphylococcus*<sup>27</sup> that is thereafter dominated by *Bifidobacterium* until the introduction of solid food and the development of adult-type micro-organisms such as those in the genera *Bacteroides*, *Prevotella*, *Ruminococcus*, *Clostridium*, and *Veillonella* (Fig. 2A). We noted with interest that the fecal samples from patients 3257RS (from a 14-month-old) and 3391RS (from a 9-month-old) in the antibiotic exposure group were atypically dominated by the bacterial family *Enterobacteriaceae* (Fig. 2A). Patient 3391 was treated with antibiotics from birth, and we postulate that the antibiotic treatment of these patients delayed the transition from the early dominance of *Enterobacteriaceae* to the flora more representative of early childhood. In differential abundance analyses, both *Bifidobacteriaceae* and *Enterobacteriaceae* were more abundant in the antibiotic treated group; however, the difference did not reach statistical significance.

There were several limitations of the current study, including small sample size. A larger study will need to be conducted to definitively assess the effects of antibiotic exposure on the urinary and GI microbiota. Additionally, we did not have temporal data for each patient showing their microbial community before antibiotic exposure and after antibiotic exposure to note individual changes in microbial composition. Finally, we cannot comment as to whether atypical urinary and/or GI microbiota observed in the antibiotic treatment group was the result of antibiotic use or alternatively a bystander effect of the congenital abnormalities present in some of the children. This will certainly be the focus of future studies.

Finally, we report an interesting case of a pediatric patient who had been on amoxicillin prophylaxis since birth and developed urosepsis after corrective surgery for UPJ obstruction. We found that the species that likely caused the urosepsis was overrepresented in the urinary microbiota of this patient prior to his surgery, and we postulate that this was due in part to the long-term antibiotic use. The implications for the utilization of more sensitive bacterial detection and profiling sequencing techniques in urinary samples may have a profound clinical impact on how clinicians approach urinary tract colonization by different bacteria.

## CONCLUSION

To date, this study provides one of the earliest characterizations of the urinary microbial community in male children. Understanding and tracking the urinary microbial community from birth through precontenance training, childhood, and pre- and postpuberty, may give us insights into the long-term implications for the development of many adult diseases. In adults, potential associations have been discovered between alterations of the urinary microbiome and development of other urologic diseases such as urinary tract infections, urgency urinary incontinence, kidney stones, etc.<sup>28-30</sup> Thus, we propose that elucidating the composition and development of the pediatric urinary

microbiome in children as well as the impact of early antibiotic exposure is an important endeavor in the field of pediatrics.

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## SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at <https://doi.org/10.1016/j.urology.2019.05.031>.

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