ORIGINAL PAPER

UROLITHIASIS

Comparison of the microbiome of bladder urine, upper urinary tract urine, and kidney stones in patients with urolithiasis

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Citation: Chorbińska J, Krajewski W, Karpiński P, et al. Comparison of the microbiome of bladder urine, upper urinary tract urine, and kidney stones in patients with urolithiasis. Cent European J Urol. 2025; doi: 10.5173/ceju.2025.0020

Article history
Submitted: Jan. 27, 2025
Accepted: Feb. 16, 2025
Published online: Apr. 28, 2025

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Introduction It is believed that bacteria can be involved in the formation of all types of stones. The aim of study was to assess the urinary microbiome in patients with urolithiasis.

Material and methods The study group included 50 patients qualified for endoscopic treatment of urinary tract stones using: ureteroscopic lithotripsy (URSL), retrograde intrarenal surgery (RIRS), percutaneous nephrolithotripsy (PCNL), endoscopic combined intrarenal surgery (ECIRS). Before the procedure, patients were asked to collect urine and stool for analysis. Urine from the upper urinary tract and stone fragments were collected intraoperatively. The research material was subjected to 16S rRNA sequencing. The chemical composition of stones was assessed using Raman spectroscopy.

Results In the urinary bladder, upper urinary tract, and kidney stone microbiomes of patients with urolithiasis the predominant bacteria identified were: *Acinetobacter, Bifidobacterium, Corynebacterium, Cutibacterium, Paracoccus, Pseudomonas, Staphylococcus* and *Streptococcus*. Further analysis showed the relative similarity of the urinary bladder and upper urinary tract microbiomes and the dissimilarity of the kidney stone microbiome. A comparison of the upper urinary tract microbiome based on the method of urine collection and a comparison of urinary bladder and upper urinary tract microbiomes based on the presence of a DJ stent prior to the procedure showed no statistically significant differences.

Conclusions The microbiome of stones differs from the microbiome of urine, which may play a role in the pathogenesis of urolithiasis. Bladder urine and upper urinary tract urine microbiomes do not differ. Therefore, bladder urine can replace upper urinary tract urine in microbiome studies.

Key Words: microbiome ‹› urolithiasis ‹› 16S rRNA sequencing ‹› kidney stones

INTRODUCTION

Urolithiasis is one of the most common urological diseases affecting up to 20% of the population. The recurrence rate of stones within five years in first-time stone formers is 26% [1]. For many years, only urease-producing bacteria, associated with the formation of struvite stones, were considered to be the bacterial etiology of urolithiasis [1]. However, patients with urolithiasis often experience concomitant urinary tract infections and often have positive urine cultures in the pre- or postoperative period, regardless of the chemical composition of the stone [2, 3]. Therefore, it is suspected that bacteria may be involved in the development of all types of stones, including non-struvite stones.

Thanks to advances in DNA sequencing, it has been shown that the urinary tract has its own endogenous

microbiome, and the dogma that urine bomust be sterile has been disproved [4]. Considering the postulated role of bacteria in the development of stones, it is believed that not one specific bacterium, but microbiome dysbiosis plays a role in the pathogenesis of urolithiasis.
Therefore, the aim of our study was to assess the urinary and stool microbiome in patients with urolithiasis and to compare bladder, upper urinary tract and stones microbiomes. Furthermore, we assessed whether there is an association between the composition of the microbiome in patients with urolithiasis and patient- and urolithiasis-related features.

MATERIAL AND METHODS

Study design and specimen collection

The study group included patients hospitalized at the University Center of Excellence in Urology in 2022–2023, qualified for endoscopic treatment of urinary tract stones using: ureteroscopic lithotripsy (URSL), retrograde intrarenal surgery (RIRS), percutaneous nephrolithotomy (PCNL) or endoscopic combined intrarenal surgery (ECIRS). Exclusion criteria included: recent/active sexually transmitted infection, recent/active urinary tract infection, use of antibiotics in the past month.
Before the procedure, patients were asked to collect urine and stool for analysis. Urine was collected from the midstream. Stool was collected using a Kałszyk stool sample collection kit (KOSOWSKI®). Intraoperatively, urine was collected from the upper urinary tract, through the ureterorendoscope during URSL and RIRS or right after percutaneous puncture during PCNL and ECIRS. Fragments of stones were also collected to analyze microbiome and its chemical composition. The biological material for microbiome analysis was stored at –80°C until DNA isolation. Stones collected for the assessment of chemical composition were stored at room temperature. The chemical composition was assessed using Raman spectroscopy.
Samples collected for microbiome analysis were divided into 4 groups: urine from the bladder (U), urine from the upper urinary tract (UT), stones (KS) and stool (S).

DNA isolation

The patient's U, UT, S samples were used to isolate bacterial DNA as previously described [5].
For KS DNA isolation, received samples were washed with filtered PBS, snap-freezed in liquid nitrogen, and crushed with the use of mortar and pestle. 200 mg of the obtained powder was treated with a DNeasy Blood & Tissue Kit (cat. no. 69506, QIAGEN). First, the sample was incubated in an ATL buffer (supplemented with proteinase K, mutanolysin and lysozyme) for 1 hour, at 37°C (with shaking). Before transfer to the column, the suspension was treated with AL buffer followed by pure ethanol. AW1 and AW2 buffers were used for washing steps and 50 μl AE buffer allowed DNA elution. All buffers necessary for the procedure were included in the kit.

DNA library preparation and sequencing

DNA library and sequencing were performed by Novogene company (China) according to their standardized procedures.
Briefly, all the DNA samples that passed the quality control were subjected to 16S rRNA library preparation. Briefly, 16S rRNA/18SrRNA/ITS genes of distinct regions (16SV4/16SV3/16SV3- V4/16SV4- V5, 18SV4/18SV9, ITS1/ITS2, ArcV4) were amplified by polymerase chain reactions (PCR). To select PCR products of the intended size, 2% agarose gel electrophoresis was performed. In the next step, the same amount of PCR products from each sample was pooled, end-repaired, A-tailed, and ligated with Illumina adapters.
Finally, to achieve the highest quality of the obtained library, it was checked with Qubit and real-time PCR for quantification, while a bioanalyzer was used for size distribution detection. Quantified libraries were pooled and sequenced on a pair-end Illumina platform to generate 250 bp paired-end raw reads.

Bioinformatic analysis

Quality control and preprocessing

Raw paired-end sequences in FASTQ format were processed in R (v4.1.2) using the dada2 package for quality control, trimming, and filtering [6]. Sequences were truncated to a fixed length of 210 bp for forward and 220 bp for reverse reads, with a maximum allowable number of expected errors set to 2 [6]. Sequences were dereplicated, and error models were learned independently for forward and reverse reads. Paired-end reads were merged, and chimeric sequences were removed. Resulting sequences were organized into an amplicon sequence variant (ASV) table.

Taxonomic assignment

The "dada2" package was used for taxonomic assignment of ASVs using the Silva v138.1 reference

database [6]. Taxonomy was assigned using the naive Bayesian classifier with a minimum bootstrap value of 80 [7]. Taxonomic tables were combined with sequence data to create a phyloseq object for further processing [8].

α- and β-diversity analysis

To evaluate microbial diversity within each subgroup, α-diversity indices, including the Chao1 richness index, Shannon diversity index, Simpson evenness index, and Gini index, were computed using the "mia" package and t-test [9]. β-diversity was assessed through principal component analysis (PCA) using the microbiome package and unsupervised clustering using "ComplexHeatmap" package [10, 11]. Briefly, data were log-transformed and visualized at the genus level, focusing on the top 50 genera across samples. Core microbiome analysis was conducted by defining prevalent taxa (present in at least 10% of samples) and aggregating rare taxa below the genus level. Relative abundances were calculated and visualized to illustrate composition patterns within each subgroup.

Confounding factors and multivariate analysis

To account for potential confounding factors, multivariate analyses were performed using PERMANOVA with the "microViz" package [12]. Confounding variables such as age, gender, BMI, and comorbidities (e.g., hypertension, diabetes) were tested for associations with microbial composition. Additional confounding analysis was performed using "swamp" package to evaluate potential clustering based on metadata variables [13].

Differential abundance and statistical testing

Differences in bacterial composition across subgroups were assessed using SIAMCAT [14]. Specific pairwise comparisons, between UT and U, KS and U, and KS and UT, were conducted to identify significantly differentially abundant genera. Differential abundance was determined using Wilcoxon rank-sum tests, and p-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. Results were visualized with association plots.
Spearman correlation was applied to assess relationships between microbial genera and selected clinical variables. The analysis focused on the top genera at the genus rank present in at least of 50% of selected samples from given subgroup (U, UT, KS or S). Taxonomic abundance data was transformed using centered log-ratio (CLR) transformation with replacing zeros with half the minimum non-zero value. A correlation heatmap was generated using the corrplot package, with correlations below an absolute value of 0.49 and FDR-adjusted p-value >0.05 masked to highlight moderate to strong correlations [15]. The modified heatmap was visualized using NMF package [16].

Bioethical standards

The study was approved by the Bioethics Committee of the Wroclaw Medical University (KB-252/2022).

RESULTS

Patient characteristics

50 patients were qualified for the study, including: 33 women (66%) and 17 men (34%), aged 23–89 years (mean 55,64). The RIRS procedure was performed most frequently (42%). Half of the patients had a double J (DJ) stent inserted before the procedure. Most of the stones (34) were composed of calcium oxalate monohydrate.
Detailed characteristics of patients is presented in Table 1.
Of the 200 samples, 175 were included in the analysis, including 50 S, 50 U, 39 UT and 36 KS. The remaining samples were excluded due to insufficient genetic material for sequencing.

General characteristics of the microbiome

In the U, UT and KS microbiomes the predominant bacteria identified were: *Acinetobacter, Bifidobacterium, Corynebacterium, Cutibacterium, Paracoccus, Pseudomonas, Staphylococcus* and *Streptococcus* (Figure 1). At the level of α-diversity, no differences were demonstrated between the groups ($p > 0.05$) (Figure 2).

Comparison of the urine from the bladder, upper urinary tract and stones microbiomes

Hierarchical clustering analysis and principal component analysis (PCA) of the microbiome showed the relative similarity of the U and UT microbiomes and the dissimilarity of the KS microbiome (Figures 3, 4).

Detailed comparison of the urine from the bladder and upper urinary tract microbiomes

As a result of statistical comparison of abundances between the UT and U subgroups, 29 genera were

found to be significantly differentially abundant between the compared groups (adjusted p-value ≤0.05) (Figure 5 and Supplementary material 1).

Detailed comparison of the stones and urine from the bladder microbiomes

A total of 83 genera were identified as significantly differentially abundant between KS and U (Figure 6 and Supplementary material 2). Genera significantly more abundant in KS included *Chryseobacterium, Brevundimonas, Microbacterium, Acidocella, Rhodococcus, Brucella*, and *Flavobacterium*. Genera enriched in U included *Reyranella, Acidovorax, Legionella, Dialister, Pajaroellobacter*, and *Sphingomonas*. Genera such as Pseudomonas and Rothia were prevalent in both KS and U.

Detailed comparison of the stones and upper urinary tract microbiomes

A statistical comparison of abundances between the KS and UT subgroups identified 63 genera with significantly differential abundance (Figure 7 and Supplementary material 3). Genera significantly more abundant in KS included *Chryseobacterium, Acidocella, Rhodococcus, Stenotrophomonas, Brevundimonas, Brucella*, and *Flavobacterium*. Genera enriched in UT included *Burkholderia–Caballeronia–Paraburkholderia, Methylobacterium–Methylorubrum, Sphingomonas*, and *Neisseria*. Genera such as *Cloacibacterium* and *Bifidobacterium* were found in both sites but were more prevalent in KS. Pseudomonas was highly prevalent in both KS and UT, with a slight reduction in UT.

Assessment of the correlation between the urine from the bladder, upper urinary tract, stones, stool microbiomes and patient-related and urolithiasis-related features

We analyzed correlations between microbiome and patient-related features such as age, weight, BMI and comorbidities and correlations between microbiome and urolithiasis-related features including stone dimensions, mean Hounsfield Units (HU), and DJ stent presence. We found no statistically significant correlations for U, UT and S subgroups. The correlation analysis of KS microbiome revealed several significant relationships (Figure 8). *Microbacterium* showed a strong positive correlation with weight (r = 0.696) and BMI (r = 0.564). *Rhodococcus* and *Brucella* exhibited a negative correlation with stone depth (r = –0.494; r = –0.571). *Methylobacterium-Methylorubrum* showed a negative correlation with stone width (r = –0.567). *Rothia* and *Flavobacterium* demonstrated positive correlations with stone width (r = 0.514) and stone depth (r = 0.520), respectively. A comparison of the UT microbiome based on the method of urine collection, whether antegrade or percutaneous and a comparison of the U and UT microbiomes based on the presence of a DJ stent prior to the procedure showed no statistically significant differences.

Table 1. *Patient characteristics*

No. of Patients	50	
Women	33	66%
Men	17	34%
Age, mean (range)	55.64	23–89
BMI, mean (range)	28.02	19.13–35.08
Comorbidities		
Obesity	8	16%
Hypertension	11	22%
Diabetes mellitus	6	12%
Dyslipidemia	1	2%
Metabolic syndrome	6	12%
Hypothyroidism	8	16%
Hyperthyroidism	1	2%
Procedure		
URSL	5	10%
RIRS	21	42%
PCNL	11	22%
ECIRS	13	26%
Stone characteristics		
Location		
Ureter	13	
Pelvis	21	
Upper calyx	5	
Middle calyx	13	
Lower calyx	24	
Staghorn	7	
Side		
Right	22	44%
Left	16	32%
Bilateral	12	24%
Size [mm], mean (range)		
Height	13.56	3.59–48.8
Width	17.56	5.16–58.36
Depth	11.97	4.4–27.6
HU, mean (range)	1,016	312–1703
DJ stent preoperatively		
Yes	25	50%
No	25	50%
Stone composition		
Uric acid	6	
Calcium oxalate monohydrate	34	
Calcium oxalate dihydrate	18	
Carbapatite	15	
Magnesium ammonium phosphate	4	
Mixed	22	

BMI – body mass index; DJ – double-J; ECIRS – endoscopic combined intrarenal surgery; HU – Hounsfield Units; PCNL – percutaneous nephrolithotripsy; RIRS – retrograde intrarenal surgery URSL – ureteroscopic lithotripsy

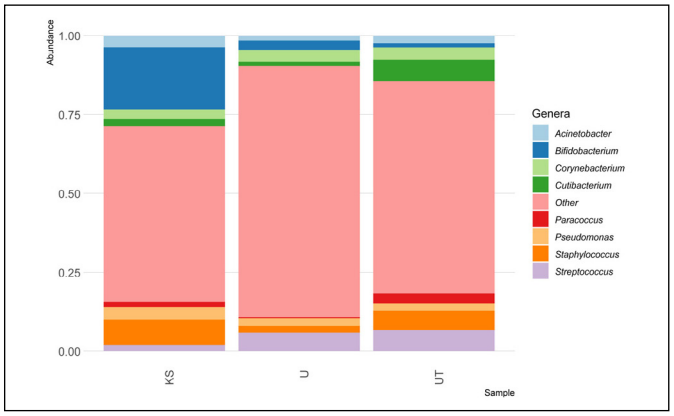

Figure 1. *Relative abundance of selected microbial genera across three subgroups: kidney stones (KS), urine (U), and urinary tract (UT). The data was averaged within each subgroup and agglomerated at the "Genus" level. Clear differences in the microbial composition are observed between the subgroups, highlighting distinct community structures in each environment. Rare taxa, defined as those present in fewer than 50% of the samples, were excluded from the analysis to focus on more prevalent genera.*

DISCUSSION

Until recently, it was believed that only urease-producing bacteria were involved in the pathogenesis of urolithiasis and are responsible for the formation of struvite stones [1]. Considering the rare occurrence of struvite stones and the common occurrence of urinary tract infections and positive urine cultures in patients with urolithiasis, it is believed that bacteria can be involved in the formation of all types of stones, including non-struvite stones [2, 3].

In our study, we assessed the U, UT, KS and S microbiomes. In the U, UT and KS microbiomes, the most abundant bacteria were *Acinetobacter, Bifidobacterium, Corynebacterium, Pseudomonas, Staphylococcus, Streptococcus, Cutibacterium*, and *Paracoccus*. These results are similar to those obtained by other authors. Dornbier et al. also assessed KS, U and UT microbiomes, and the dominant taxa were *Staphylococcus, Streptococcus, Corynebacterium, Bifidobacterium*, as well as *Veillonella, Haemophilus, Proteus, Lactobacillus*, and

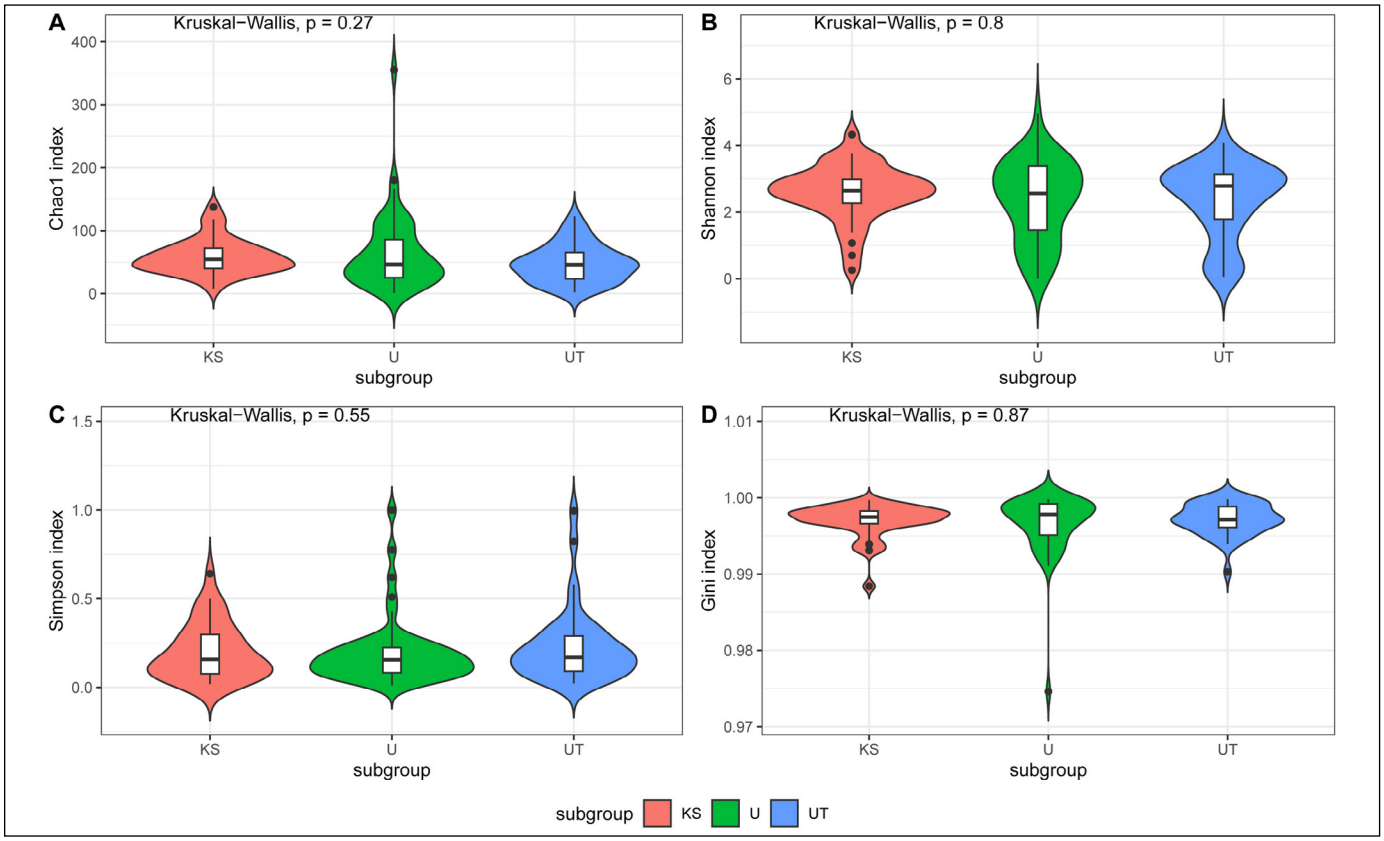

Figure 2. *The major α-diversity measures in the analysis of the V3-V4 16S region in KS (stones), U (urinary bladder), and UT (upper urinary tract). α-iversity measures* **(A)** *Chao1 index,* **B)** *Shannon index,* **C)** *Simpson index, and* **D)** *Gini index) rep-resenting key aspects of microbial diversity, including species richness, evenness, and overall diversity. Each plot displays the distribution of diversity indices across different sample groups. No significant differences were detected among groups for any of the diversity measures, as determined by the Kruskal-Wallis test (p >0.05).*

the *Enterobacteriaceae* family [17]. Liu et al. [18] compared the U and UT microbiomes. They found a high prevalence of the genera *Acinetobacter, Bifidobacterium, Corynebacterium, Staphylococcus, Streptococcus*, as well as *Delftia, Propionibacterium, Pontibacter, Sphingomonas*, and *Prevotella*. The high prevalence of *Acinetobacter, Pseudomonas*, and *Staphylococccus* in U and KS microbiomes was also described by Hong et al. [2] and Tavichakorntrakool et al. [19]. Xie et al. [20] reported a higher prevalence of the genus *Acinetobacter*.

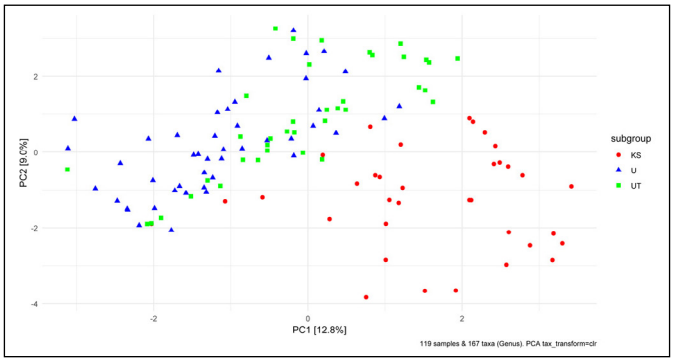

Figure 3. *Principal coordinate analysis (PCoA) of microbiome data from 125 samples across three subgroups: kidney stones (KS), urine (U), and urinary tract (UT). The PCoA plot shows clear separation of the KS subgroup, which forms a distinct cluster, while some U and majority of UT samples are more closely grouped. Intra-variability between the U and UT samples is much lower than the intra-variability between the KS samples.*

By comparing the U and UT microbiomes, we demonstrated their relative similarity. Only detailed analysis revealed differences in the abundance of some bacteria genera and the occurrence of a group of genera only in the U microbiome. These differences may result from improper urine collection by the patient. In addition, in our study, we did not demonstrate that the method of collecting urine from the renal pelvis, thorough the ureterorenoscope or percutaneously, had an effect on the composition of the UT microbiome. To our knowledge, this is the first study first study to assess this aspect. Other authors have also shown no differences between U and UT microbiomes [17, 18, 20]. Liu et al. [18], in order to minimize the risk of the influence of bacteria from the bladder performed bladder disinfection with iodophor before collecting urine from the renal pelvis. They also did not show any differences in the U and UT microbiomes. Therefore, it can be assumed that U is representative and can replace UT in microbiome studies.

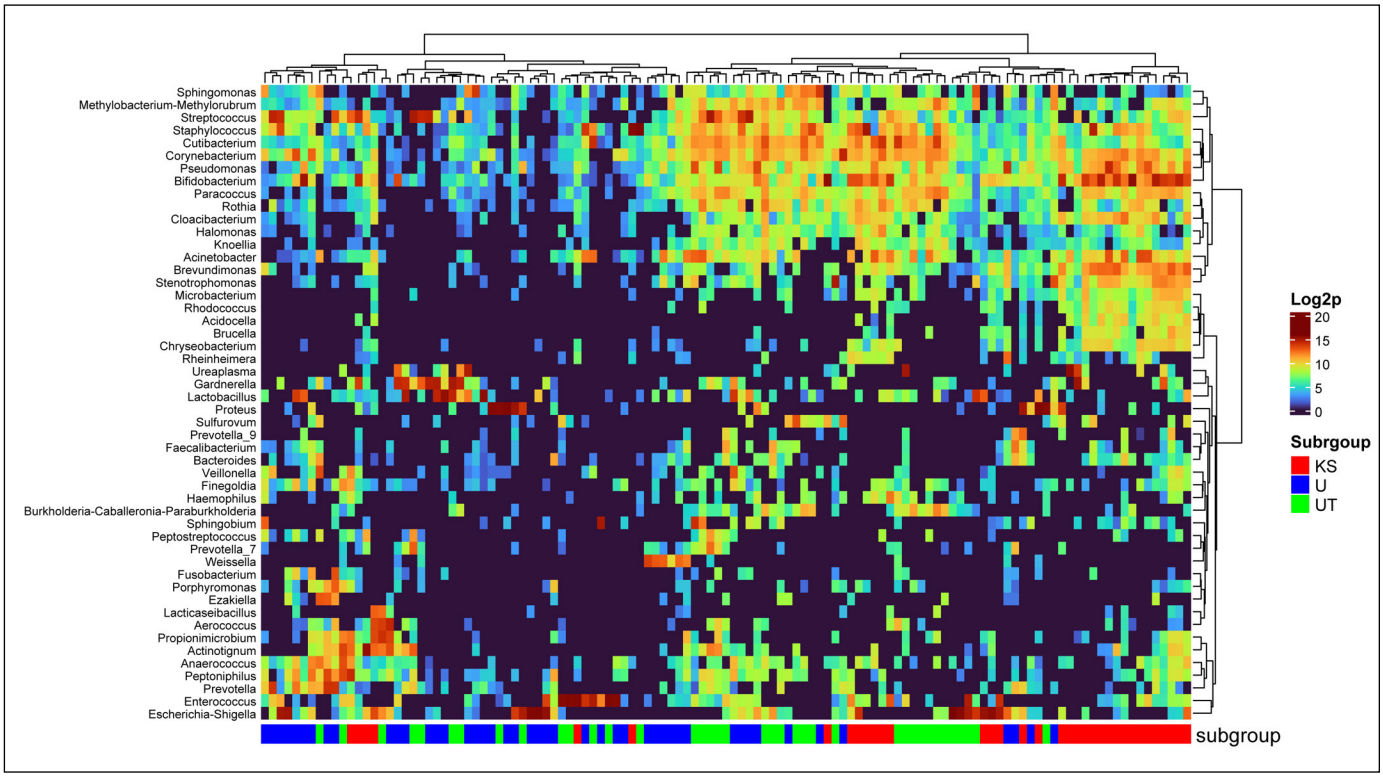

Figure 4. *Hierarchical clustering of microbiome data from 125 samples across three subgroups: stones (KS), urinary bladder (U), and upper urinary tract (UT). The dendrogram demonstrates that the KS subgroup forms a distinct cluster, while samples from the U and UT subgroups are intermixed, indicating a closer similarity between these two sample types.*

It allows for better comparison with a healthy controls in further studies, because collecting urine from the renal pelvis in healthy controls raises significant ethical concerns.

Comparison of the effect of the presence of a DJ stent on urinary microbiome also did not show any significant differences. In our study, a DJ stent was inserted 50% of patients preoperatively. In the study by Dornbier et al. [17], it was placed in 96.1% of patients at the time of stone extraction. They did not show any differences in the U, UT and KS microbiomes. In the study by Xie et al. [20], the presence of a ureteral stent preoperatively was an exclusion criterion. Again, no differences were found between the U and UT microbiomes. Buhmann et al. assessed the microbiome of ureteral stents placed 3 to 6 weeks after treatment for urolithiasis. The most common genera included *Actinomyces, Staphylococcus, Streptococcus, Corynebacterium, Lactobacillus, Achromobacter, Facklamia, Anaerococcus, Gardnerella, Atopobium, Actinotignum*, and the *Enterobacteriaceae* family [21]. The composition of the ureteral stent microbiome partially overlaps with the dominant taxa observed in our study. It therefore appears that the presence of the DJ stent may not affect the composition of the microbiome and that its microbiome does not differ from the U microbiome, but further studies are needed to confirm this thesis.

We also showed that KS microbiome differs from U and UT microbiomes. KS microbiome was more abundant in the genera *Chryseobacterium, Brevundimonas, Microbacterium, Acidocella, Rhodococcus, Brucella, Flavobacterium*, and *Stenotrophomonas*. These results are different from those reported by other authors. Neither Lemberger et al. [17] nor Dornbier et al. [22] showed differences between

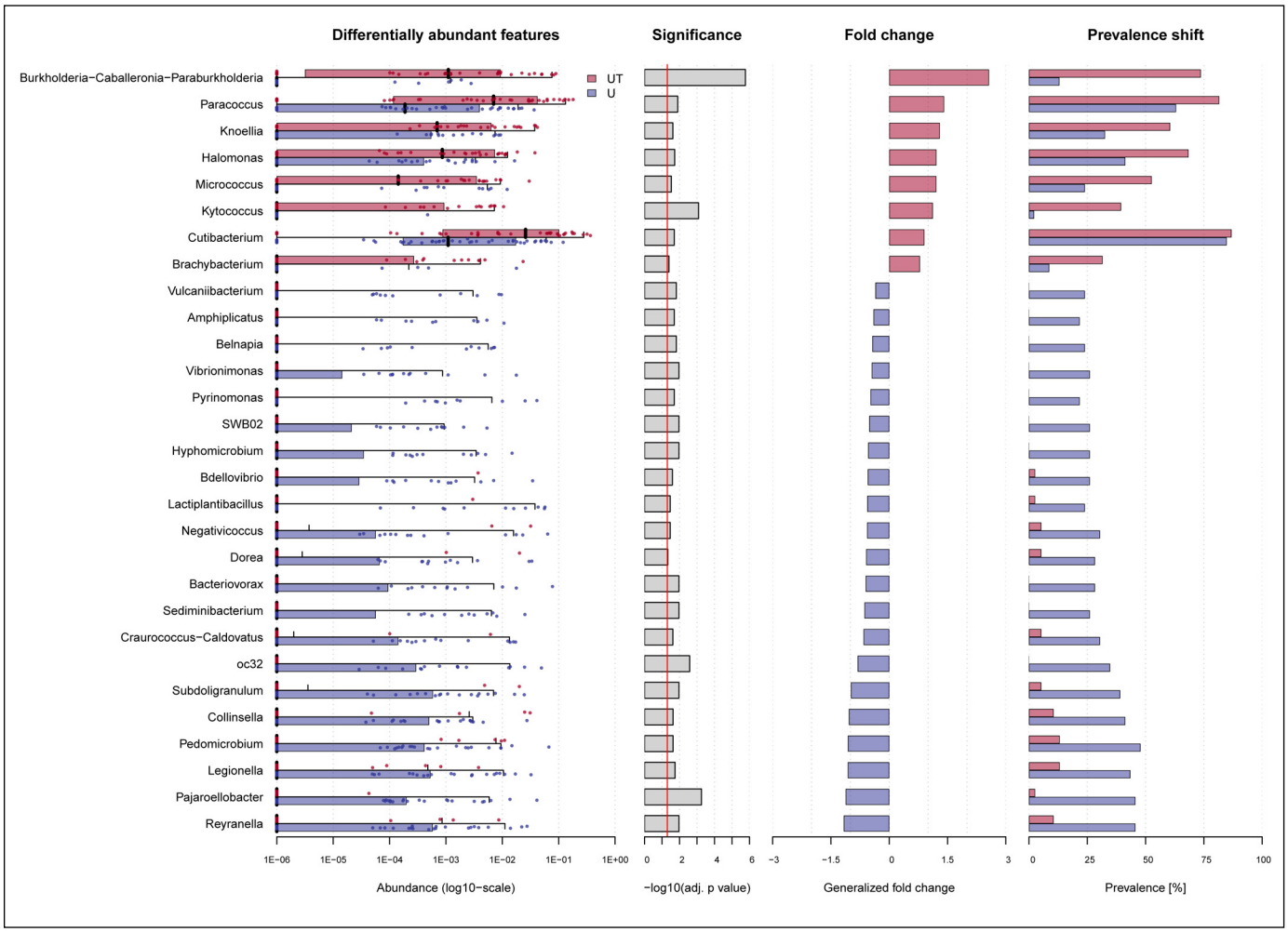

Figure 5. *Association between bacterial genera agglomerated to the "Genus" level and phenotype classes: upper urinary tract (UT) vs. urinary bladder (U). The plot displays the log10-transformed abundances of each genus for both phenotype classes (UT and U) from left to right. Statistical associations are determined by an adjusted p-value (≤0.05) using the Wilcoxon test. Additionally, generalized fold change and prevalence shift between the two classes are shown.*

KS microbiome and U and UT microbiomes, only Dornbier et al. showed that KS microbiome was enriched in dominant taxa compared to the U microbiome. There is a hypothesis that bacteria responsible for stone formation are located in the stone nidus, while bacteria responsible for urinary tract infections in the course of urolithiasis secondarily cover the surface of the stone [2]. Our results seem to confirm this hypothesis. However, it is necessary to perform more studies with a detailed analysis of the stone microbiome from samples taken from different parts of the stone.

It is suspected that specific types of bacteria may be responsible for the development of specific types of urinary stones. Our research, as well as the study by Lemberger et al. [22] did not show such a relationship. However, in most studies, the dominant type of deposit is calcium oxalate, which makes it difficult to assess the relationship between the microbiome of U and KS and the formation of rarer types of non-struvite stones.

It is believed that risk factors for the development of urolithiasis may promote the development of urolithiasis by modifying the composition of the urinary microbiome [23]. However, we failed to demonstrate a relationship between the U, UT, KS, S microbiomes and almost all patient- and urolithiasis-related features. A similar lack of relationship has been reported by other authors [18, 22, 23]. We only showed correlations between the KS microbiome and patient weight and BMI and between the KS microbiome and stone dimensions. On this basis, we hypothesize that the composition of the stone microbiome may change with increasing stone dimensions. However, these results do not allow drawing broad conclusions, but they may suggest a direction for further research.

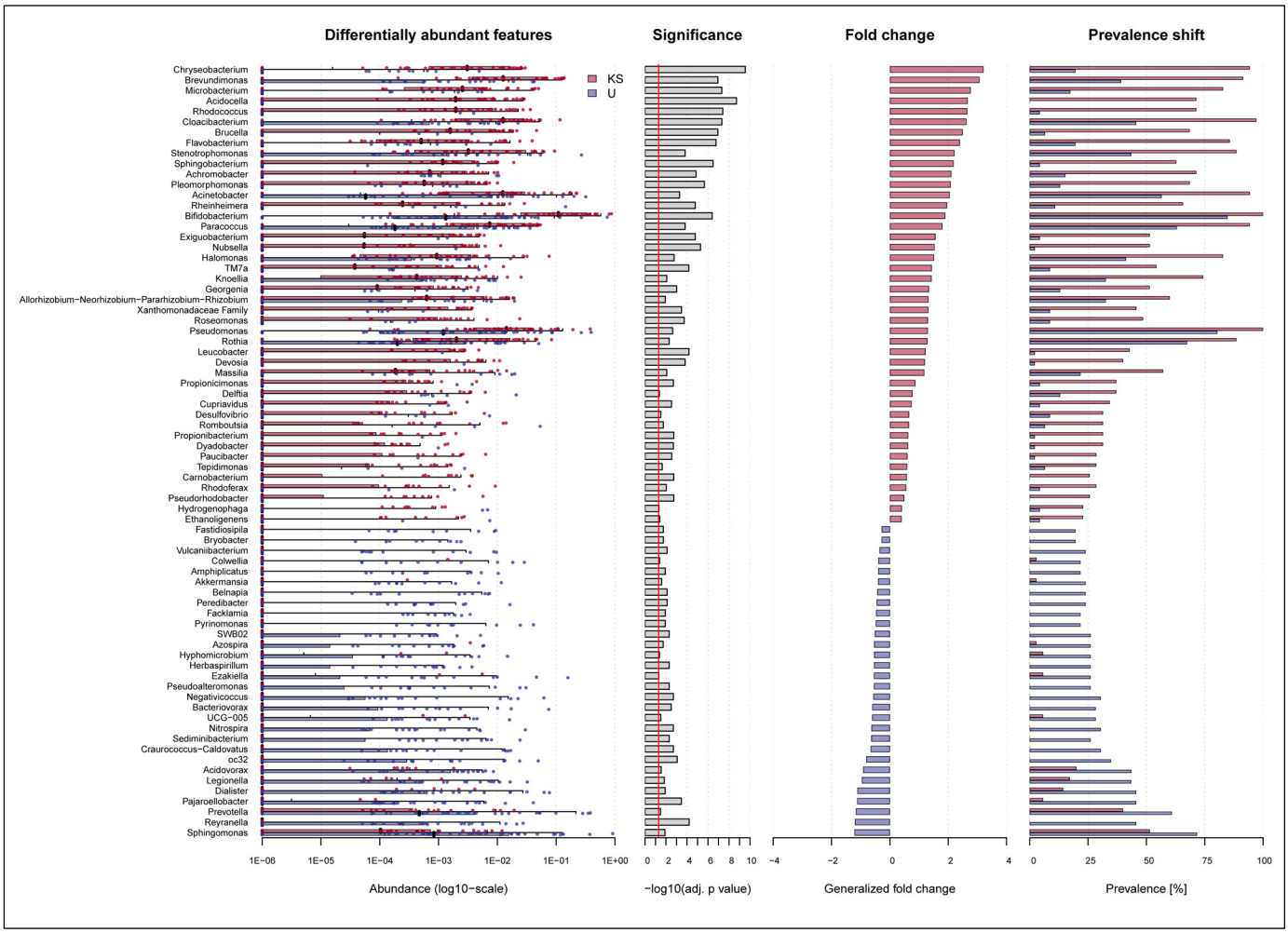

Figure 6. *Association between bacterial genera agglomerated to the "Genus" level and phenotype classes: kidney stones (KS) vs urinary bladder (U). The plot displays the log10-transformed abundances of each genus for both phenotype classes (KS and U) from left to right. Statistical associations are determined by an adjusted p-value (≤0.05) using the Wilcoxon test. Additionally, generalized fold change and prevalence shift between the two classes are shown.*

Our work has several limitations. First, the study group was small and single-center due to the costs of sequencing. We hope to conduct a multicenter study with a larger cohort in the future. Secondly, we did not include a control group in the study. This is due to the lack of possibility of noninvasive collection of urine from the upper urinary tract in healthy volunteers. We revealed that U and UT microbiomes do not differ significantly, which will allow the use of bladder urine from healthy controls for comparison in subsequent studies. Thirdly, collection of stone samples using a ureterorenoscope involves its passage through the urinary tract, which carries a risk of sample contamination. It may be limited by the routine use of ureteral access sheaths in further studies. Fourthly, we were able to obtain enough genetic material for sequencing only in 36 stones, which limits the possibility of comparing different types of stones. Fifthly, 16S rRNA sequencing does not detect microorganisms other than bacteria and archaea, such as fungi or viruses, which may play a role in the onset of the disease. Finally, like most microbiome studies, the study was descriptive in nature, which prevents us from establishing a causal relationship between the urinary microbiome and urolithiasis. Further studies are needed to determine whether changes in the urinary microbiome are involved in the pathogenesis of urolithiasis or are a consequence of the development of stones.

CONCLUSIONS

In conclusion, we compared the microbiomes of bladder urine, upper urinary tract urine, stones and stool in patients with urolithiasis. We showed that the stone microbiome differs from urine microbiome, which may play a role in the pathogenesis

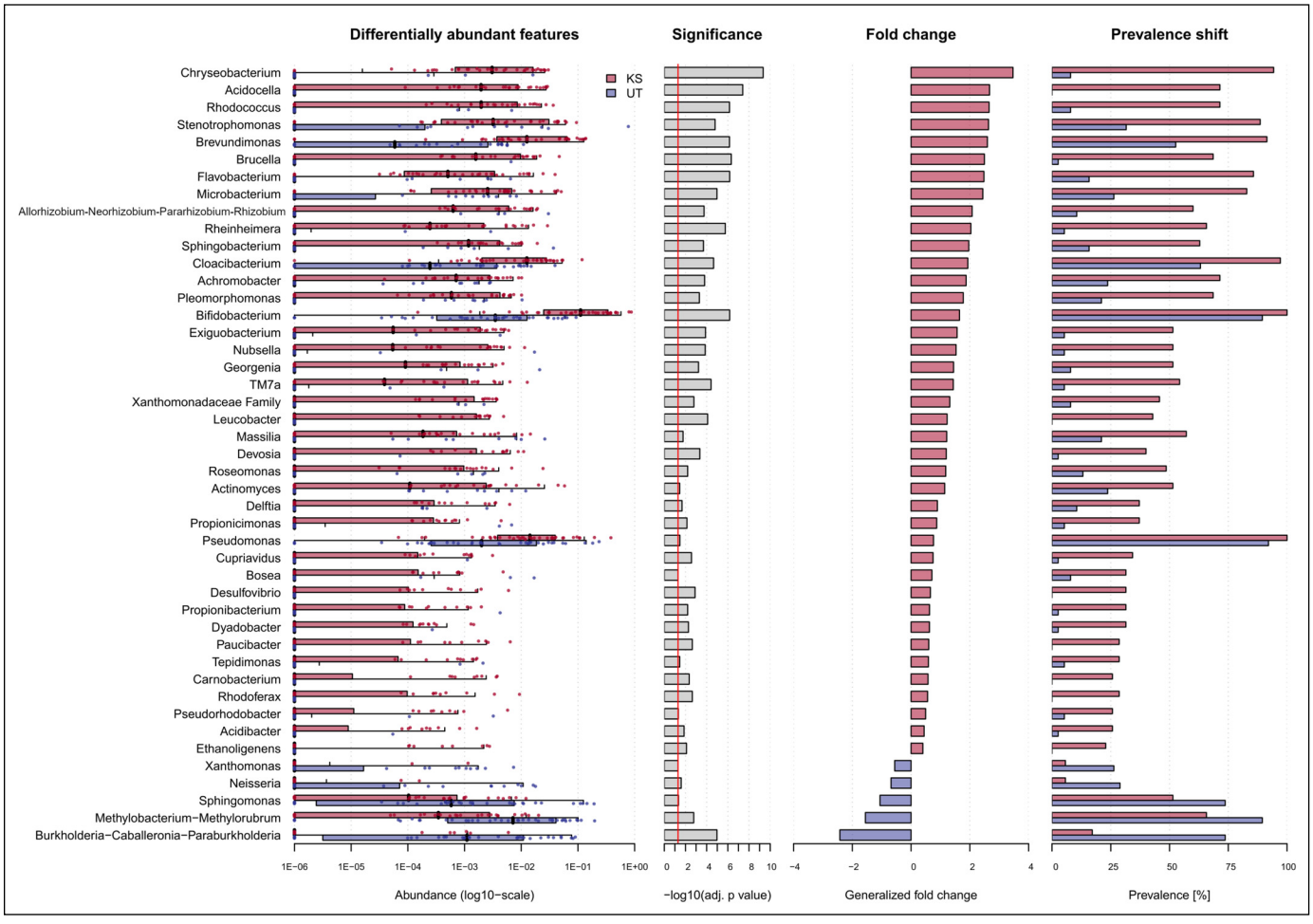

Figure 7. *Association between bacterial genera agglomerated to the "Genus" level and phenotype classes: kidney stones (KS) vs upper urinary tract (UT). The plot displays the log10-transformed abundances of each genus for both phenotype classes (KS and UT) from left to right. Statistical associations are determined by an adjusted p-value (≤0.05) using the Wilcoxon test. Additionally, generalized fold change and prevalence shift between the two classes are shown.*

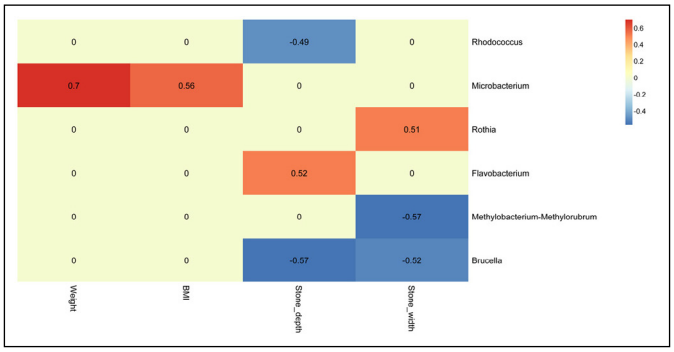

Figure 8. *Heatmap showing significant correlations between microbial taxa and clinical variables in kidney stone (KS) samples. The color intensity represents the strength and direction of the correlation, with positive correlations in shades of red and negative correlations in shades of blue. Correlations are displayed only for values with an absolute correlation coefficient ≥0.49 and an adjusted p-value <0.05. Numeric values within the heatmap cells indicate the rounded correlation coefficients. Rows represent microbial taxa, while columns correspond to clinical variables.*

of urolithiasis. In addition, we showed no effect of the presence of the DJ stent on the composition of the microbiome. Further studies are necessary on a larger cohort on this topic are necessary to confirm these results. Moreover, the comparison of bladder urine and upper urinary tract microbiomes showed their relative similarity. Therefore, it can be assumed that bladder urine is representative and can replace upper urinary tract urine in microbiome studies.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

FUNDING

The study was carried out as part of the Young Scientists grant (SUBK.C090.22.066).

ETHICS APPROVAL STATEMENT

The study was approved by the Bioethics Committee of the Wroclaw Medical University (KB-252/2022).

SUPPLEMENTARY MATERIALS

Supplementary material 1. Detailed comparison of the urine from the bladder and upper urinary tract microbiomes

As a result of statistical comparison of abundances between the UT and U subgroups, 29 genera were found to be significantly differentially abundant between the compared groups (adjusted p-value ≤ 0.05) (Suppl. Table 1). The comparison showed that the genera *Brachybacterium, Burkholderia–Caballeronia–Paraburkholderia, Cutibacterium, Halomonas, Knoellia, Micrococcus* and *Paracoccus* were present in both subgroups, but their abundances were significantly higher in the UT group reaching prevalences above 50%. In addition, *Kytococcus was almost exclusively present in UT group reaching 39% of prevalence*. Large group of 15 genera (*Vulcaniibacterium, Amphiplicatus, Belnapia, Vibrionimonas, Pyrinomonas, SWB02, Hyphomicrobium, Bdellovibrio, Lactiplantibacillus, Negativicoccus, Dorea, Bacteriovorax, Sediminibacterium, Craurococcus–Caldovatus,* oc32 and *Pajaroellobacter*) were almost exclusively present in up to 25% of U samples. The remaining 5 genera, including *Reyranella, Legionella, Pedomicrobium, Collinsella* and *Subdoligranulum*, were predominantly present in U samples (approx. 40% of U samples), with only a few instances in the UT group.

Supplementary material 2. Detailed comparison of the stones and urine from the bladder microbiomes

A total of 83 genera were identified as significantly differentially abundant between KS and U (Suppl. Table 2). Genera significantly more abundant in KS included *Chryseobacterium, Brevundimonas, Microbacterium, Acidocella, Rhodococcus, Brucella,* and *Flavobacterium*. *Chryseobacterium* showed the highest fold change (3.21) and was present in 94.29% of KS samples compared to 19.57% in U, while *Brevundimonas*, with a fold change of 3.07, was prevalent in 91.43% of KS samples vs 39.13% in U. *Acidocella* was exclusive to KS with a prevalence of 71.43% and a fold change of 2.67, and *Rhodococcus* had a fold change of 2.65 and minimal presence in U (4.35%). *Brucella* (fold change = 2.49) and *Flavobacterium* (fold change = 2.40) also demonstrated higher prevalence in KS samples compared to U. Genera enriched in U included *Reyranella, Acidovorax, Legionella, Dialister, Pajaroellobacter,* and *Sphingomonas*. *Reyranella*, with a fold change of –1.20, was exclusive to U (prevalence 45.65%) and absent in KS. *Pajaroellobacter* (fold change = –1.12) and *Dialister* (fold change = –1.11) were significantly more prevalent in U (45.65%) but nearly absent in KS (5.71% and

14.29%, respectively). *Acidovorax* (fold change = –0.92) and *Legionella* (fold change = –0.96) were more abundant in U, with prevalences of 43.48% and 43.48% in U compared to 20.00% and 17.14% in KS, respectively. Genera such as *Pseudomonas* and *Rothia* were prevalent in both KS and U. *Pseudomonas*, with a fold change of 1.30, was found in all KS samples and most U samples (80.43%), while *Rothia* had a fold change of 1.29, with a prevalence of 88.57% in KS and 67.39% in U, showing their shared presence across the two environments with differing abundances.

Supplementary material 3. Detailed comparison of the stones and upper urinary tract microbiomes

A statistical comparison of abundances between the KS and UT subgroups identified 63 genera with significantly differential abundance (Suppl. Table 3). Genera significantly more abundant in KS included *Chryseobacterium, Acidocella, Rhodococcus, Stenotrophomonas, Brevundimonas, Brucella*, and *Flavobacterium*. Among these, *Chryseobacterium* had the highest fold change (3.47) with a prevalence of 94.29%

Suppl. Table 1. *Differentially abundant genera identified between urinary tract (UT) and urinary bladder (U) samples. Fold change values represent the log-transformed differences in abundance between the two sites, with positive values indicating higher abundance in UT and negative values indicating higher abundance in U. Prevalence values denote the proportion of samples in which a genus was detected in UT and U. Statistical significance of differences is indicated by adjusted p-values (p.adj). Only genera with significant differential abundance (p.adj < 0.05) are included*

Genera	Fold change	P.adj	Prevalence in UT	Prevalence in U
Burkholderia-Caballeronia-Paraburkholderia	2.565633892	1.72098E-06	0.736842105	0.130434783
Paracoccus	1.411790319	0.012691747	0.815789474	0.630434783
Knoellia	1.301327126	0.024068651	0.605263158	0.326086957
Halomonas	1.214140487	0.018947631	0.684210526	0.413043478
Micrococcus	1.211317282	0.029433537	0.526315789	0.239130435
Kytococcus	1.121385489	0.000814289	0.394736842	0.02173913
Cutibacterium	0.90135918	0.020088907	0.868421053	0.847826087
Brachybacterium	0.791113444	0.039981511	0.315789474	0.086956522
Vulcaniibacterium	–0.356003071	0.015217793	0	0.239130435
Amphiplicatus	–0.404596323	0.020088907	0	0.217391304
Belnapia	–0.433585296	0.015217793	0	0.239130435
Vibrionimonas	–0.449081028	0.010847507	0	0.260869565
Pyrinomonas	–0.482612415	0.020088907	0	0.217391304
SWB02	–0.516473493	0.010847507	0	0.260869565
Hyphomicrobium	–0.541708916	0.010847507	0	0.260869565
Bdellovibrio	–0.551736892	0.02559233	0.026315789	0.260869565
Lactiplantibacillus	–0.565887784	0.034087007	0.026315789	0.239130435
Negativicoccus	–0.568884428	0.033934981	0.052631579	0.304347826
Dorea	–0.594983496	0.046664474	0.052631579	0.282608696
Bacteriovorax	–0.606984377	0.010847507	0	0.282608696
Sediminibacterium	–0.639978722	0.010847507	0	0.260869565
Craurococcus–Caldovatus	–0.660770332	0.024068651	0.052631579	0.304347826
oc32	–0.812495577	0.002630734	0	0.347826087
Subdoligranulum	–0.987443855	0.010847507	0.052631579	0.391304348
Collinsella	–1.037913004	0.023463593	0.105263158	0.413043478
Pedomicrobium	–1.064147922	0.023463593	0.131578947	0.47826087
Legionella	–1.067759796	0.017870127	0.131578947	0.434782609
Pajaroellobacter	–1.121239796	0.000554967	0.026315789	0.456521739
Reyranella	–1.170141567	0.010847507	0.105263158	0.456521739

in KS and 7.89% in UT. *Acidocella* was exclusive to KS (71.43%, fold change = 2.67), while *Rhodococcus* showed high prevalence in KS (71.43%, fold change = 2.65) and minimal presence in UT (7.89%). Brucella and Flavobacterium were also predominantly found in KS with fold changes of 2.50 and 2.48, respectively. Genera enriched in UT included *Burkholderia–Caballeronia–Paraburkholderia, Methylobacterium–Methylorubrum, Sphingomonas*, and *Neisseria*. *Burkholderia–Caballeronia–Paraburkholderia* had the highest negative fold change (–2.42), with a prevalence of 73.68% in UT compared to 17.14% in KS.

Suppl. Table 2. *Differentially abundant genera identified between kidney stone (KS) and urinary bladder (U) samples. Fold change values indicate the log-transformed differences in abundance between the two sites, with positive values indicating genera more abundant in KS and negative values indicating genera more abundant in U. Prevalence values represent the proportion of samples in which each genus was detected in KS and U. Statistical significance of differences is represented by adjusted p-values (p.adj). Only genera with significant differential abundance (p.adj < 0.05) are included in the table*

Genera	Fold change	Prevalence in KS	Prevalence in U	p.adj
Chryseobacterium	3.2116705	0.942857143	0.195652174	2.85507E-10
Brevundimonas	3.073119547	0.914285714	0.391304348	1.14766E-07
Microbacterium	2.772512469	0.828571429	0.173913043	4.84005E-08
Acidocella	2.668013939	0.714285714	0	1.93947E-09
Rhodococcus	2.653689711	0.714285714	0.043478261	3.88317E-08
Cloacibacterium	2.625373344	0.971428571	0.456521739	4.76809E-08
Brucella	2.494321134	0.685714286	0.065217391	1.14766E-07
Flavobacterium	2.397236254	0.857142857	0.195652174	1.7065E-07
Stenotrophomonas	2.212614186	0.885714286	0.434782609	0.000150813
Sphingobacterium	2.186430443	0.628571429	0.043478261	3.36807E-07
Achromobacter	2.105979253	0.714285714	0.152173913	1.38854E-05
Pleomorphomonas	2.085858923	0.685714286	0.130434783	2.25464E-06
Acinetobacter	2.051382239	0.942857143	0.565217391	0.000506055
Rheinheimera	1.958520646	0.657142857	0.108695652	1.61725E-05
Bifidobacterium	1.899770835	1	0.847826087	3.96337E-07
Paracoccus	1.802514783	0.942857143	0.630434783	0.000150813
Exiguobacterium	1.56390584	0.514285714	0.043478261	1.61725E-05
Nubsella	1.52999373	0.514285714	0.02173913	5.13849E-06
Halomonas	1.51508167	0.828571429	0.413043478	0.001678905
TM7a	1.434934312	0.542857143	0.086956522	6.72138E-05
Knoellia	1.429505908	0.742857143	0.326086957	0.00843816
Georgenia	1.338523685	0.514285714	0.130434783	0.000964618
Allorhizobium–Neorhizobium–Pararhizobiu–Rhizobium	1.318521377	0.6	0.326086957	0.011648865
Xanthomonadaceae family	1.316212	0.457142857	0.086956522	0.000337856
Roseomonas	1.302796164	0.485714286	0.086956522	0.000182306
Pseudomonas	1.302463828	1	0.804347826	0.002349899
Rothia	1.291573524	0.885714286	0.673913043	0.004913594
Leucobacter	1.225735993	0.428571429	0.02173913	6.51178E-05
Devosia	1.196823852	0.4	0.02173913	0.000150813
Massilia	1.180130774	0.571428571	0.217391304	0.00843816
Propionicimonas	0.870494661	0.371428571	0.043478261	0.001993679
Delftia	0.774295689	0.371428571	0.130434783	0.042655307

Suppl. Table 2. *Conntinued*

Genera	Fold change	Prevalence in KS	Prevalence in U	p.adj
Cupriavidus	0.747291896	0.342857143	0.043478261	0.002989659
Desulfovibrio	0.657012229	0.314285714	0.086956522	0.03113351
Romboutsia	0.654557733	0.314285714	0.065217391	0.017621913
Propionibacterium	0.628356832	0.314285714	0.02173913	0.001810523
Dyadobacter	0.625164731	0.314285714	0.02173913	0.001974176
Paucibacter	0.600618193	0.285714286	0.02173913	0.002867887
Tepidimonas	0.590020876	0.285714286	0.065217391	0.0223693
Carnobacterium	0.576140056	0.257142857	0	0.001810523
Rhodoferax	0.561642398	0.285714286	0.043478261	0.00940123
Pseudorhodobacter	0.494305972	0.257142857	0	0.001810523
Hydrogenophaga	0.409733591	0.228571429	0.043478261	0.046513227
Ethanoligenens	0.401709452	0.228571429	0.043478261	0.039223595
Fastidiosipila	–0.280427475	0	0.195652174	0.017745457
Bryobacter	–0.282419335	0	0.195652174	0.017745457
Vulcaniibacterium	–0.354612871	0	0.239130435	0.007834667
Colwellia	–0.393239838	0.028571429	0.217391304	0.040620772
Amphiplicatus	–0.401065647	0	0.217391304	0.011648865
Akkermansia	–0.402783446	0.028571429	0.239130435	0.025959845
Belnapia	–0.432100549	0	0.239130435	0.007834667
Peredibacter	–0.455240232	0	0.239130435	0.007834667
Facklamia	–0.473596826	0	0.217391304	0.011648865
Pyrinomonas	–0.482985906	0	0.217391304	0.011648865
SWB02	–0.5146612	0	0.260869565	0.004913594
Azospira	–0.537626956	0.028571429	0.260869565	0.018588907
Hyphomicrobium	–0.538716603	0.057142857	0.260869565	0.041688167
Herbaspirillum	–0.540555985	0	0.260869565	0.004913594
Ezakiella	–0.54544366	0.057142857	0.260869565	0.048607431
Pseudoalteromonas	–0.545622026	0	0.260869565	0.004913594
Negativicoccus	–0.566830451	0	0.304347826	0.001993679
Bacteriovorax	–0.605209909	0	0.282608696	0.003148905
UCG-005	–0.607679005	0.057142857	0.282608696	0.031288463
Nitrospira	–0.632520613	0	0.304347826	0.001993679
Sediminibacterium	–0.638539303	0	0.260869565	0.004913594
Craurococcus–Caldovatus	–0.658972973	0	0.304347826	0.001993679
oc32	–0.807842299	0	0.347826087	0.000866322
Acidovorax	–0.915988732	0.2	0.434782609	0.028438662
Legionella	–0.959035061	0.171428571	0.434782609	0.014560994
Dialister	–1.113775496	0.142857143	0.456521739	0.012132818
Pajaroellobacter	–1.119407187	0.057142857	0.456521739	0.000341973
Prevotella	–1.167234465	0.4	0.608695652	0.03312955
Reyranella	–1.199857463	0	0.456521739	6.21782E-05
Sphingomonas	–1.214094912	0.514285714	0.717391304	0.012540092

Suppl. Table 3. *Differentially abundant genera identified between kidney stone (KS) and urinary tract (UT) samples. Fold change values indicate the log-transformed differences in abundance between the two sites, with positive values indicating genera more abundant in KS and negative values indicating genera more abundant in UT. Prevalence values represent the proportion of samples in which each genus was detected in KS and UT. Statistical significance of differences is represented by adjusted p-values (p.adj). Only genera with significant differential abundance (p.adj < 0.05) are included in the table*

Genera	Fold change	Prevalence in KS	Prevalence in UT	p.adj
Chryseobacterium	3.46688663	0.942857143	0.078947368	4.63575E-10
Acidocella	2.669233421	0.714285714	0	3.89843E-08
Rhodococcus	2.654898832	0.714285714	0.078947368	7.00224E-07
Stenotrophomonas	2.636540999	0.885714286	0.315789474	1.59025E-05
Brevundimonas	2.597381169	0.914285714	0.526315789	7.00224E-07
Brucella	2.495811373	0.685714286	0.026315789	4.78393E-07
Flavobacterium	2.479236113	0.857142857	0.157894737	6.7113E-07
Microbacterium	2.440391343	0.828571429	0.263157895	1.05557E-05
Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium	2.077356841	0.6	0.105263158	0.000177466
Rheinheimera	2.034651026	0.657142857	0.052631579	1.72676E-06
Sphingobacterium	1.963246636	0.628571429	0.157894737	0.000200044
Cloacibacterium	1.930996987	0.971428571	0.631578947	2.21467E-05
Achromobacter	1.873136492	0.714285714	0.236842105	0.000156058
Pleomorphomonas	1.776893022	0.685714286	0.210526316	0.000491392
Bifidobacterium	1.646609985	1	0.894736842	6.7113E-07
Exiguobacterium	1.564856939	0.514285714	0.052631579	0.000128011
Nubsella	1.531127884	0.514285714	0.052631579	0.000136317
Georgenia	1.445199443	0.514285714	0.078947368	0.000585522
TM7a	1.436217791	0.542857143	0.052631579	3.86037E-05
Xanthomonadaceae family	1.31691846	0.457142857	0.078947368	0.001615258
Leucobacter	1.226174159	0.428571429	0	8.1207E-05
Massilia	1.212481214	0.571428571	0.210526316	0.016924381
Devosia	1.197100504	0.4	0.026315789	0.000439779
Roseomonas	1.17808901	0.485714286	0.131578947	0.006211497
Actinomyces	1.145635818	0.514285714	0.236842105	0.034653306
Delftia	0.896119648	0.371428571	0.105263158	0.021230122
Propionicimonas	0.870995787	0.371428571	0.052631579	0.007233498
Pseudomonas	0.762825183	1	0.921052632	0.033595977
Cupriavidus	0.747642846	0.342857143	0.026315789	0.002704921
Bosea	0.708675731	0.314285714	0.078947368	0.049900237
Desulfovibrio	0.657349077	0.314285714	0	0.001242685
Propionibacterium	0.628592401	0.314285714	0.026315789	0.006211497
Dyadobacter	0.625704984	0.314285714	0.026315789	0.005222672
Paucibacter	0.601058341	0.285714286	0	0.0022685
Tepidimonas	0.59089744	0.285714286	0.052631579	0.034653306
Carnobacterium	0.576566802	0.257142857	0	0.004456682
Rhodoferax	0.562161029	0.285714286	0	0.0022685
Pseudorhodobacter	0.495026862	0.257142857	0.052631579	0.047436718
Acidibacter	0.444519037	0.257142857	0.026315789	0.01364999
Ethanoligenens	0.401882603	0.228571429	0	0.007908786

Suppl. Table 3. *Conntinued*

Genera	Fold change	Prevalence in KS	Prevalence in UT	p.adj
Xanthomonas	–0.558094406	0.057142857	0.263157895	0.049900237
Neisseria	–0.683164107	0.057142857	0.289473684	0.025411934
Sphingomonas	–1.057472563	0.514285714	0.736842105	0.045896812
Methylobacterium–Methylorubrum	–1.554175049	0.657142857	0.894736842	0.001666787
Burkholderia–Caballeronia–Paraburkholderia	–2.424046693	0.171428571	0.736842105	1.05557E-05

Methylobacterium–Methylorubrum was also more abundant in UT (89.47%, fold change = –1.55) but less prevalent in KS (65.71%). *Sphingomonas* showed higher prevalence in UT (73.68%, fold change = –1.06) than in KS (51.43%), while *Neisseria* was significantly more common in UT (28.95%) compared to KS (5.71%). Genera such as *Cloacibacterium* and *Bifidobacterium* were found in both sites but were more prevalent in KS, with *Cloacibacterium* showing a prevalence of 97.14% in KS and 63.16% in UT, and *Bifidobacterium* universally present in KS and UT but slightly reduced in UT (89.47%). *Pseudomonas* was highly prevalent in both KS and UT, with a slight reduction in UT (92.11%, fold change = 0.76).

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