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Pyuria Is Associated with Dysbiosis of the Urinary Microbiota in Type 2 Diabetes Patients Receiving Sodium–Glucose Cotransporter 2 Inhibitors

Hsueh-Ju Lin [1,†], Han-Ni Chuang [1,†], Pei-Pei Jhan [1], Han-Yu Ye [1], I-Te Lee [2,3,4,*], Tzu-Hung Hsiao [1,5,6,*] and Po-Yu Liu [7,8,9,*]

1 Department of Medical Research, Taichung Veterans General Hospital, Taichung 407204, Taiwan
2 Department of Medicine, Division of Endocrinology and Metabolism, Taichung Veterans General Hospital, Taichung 40705, Taiwan
3 School of Medicine, National Yang-Ming Chiao Tung University, Taipei 11221, Taiwan
4 School of Medicine, Chung Shan Medical University, Taichung 40201, Taiwan
5 Department of Public Health, College of Medicine, Fu Jen Catholic University, New Taipei City 242062, Taiwan
6 Institute of Genomics and Bioinformatics, National Chung Hsing University, Taichung 40227, Taiwan
7 Department of Internal Medicine, Division of Infectious Diseases, Taichung Veterans General Hospital, Taichung 407204, Taiwan
8 Rong Hsing Research Center for Translational Medicine, National Chung Hsing University, Taichung 40764, Taiwan
9 Ph.D. Program in Translational Medicine, National Chung Hsing University, Taichung 40227, Taiwan
* Correspondence: itlee@vghtc.gov.tw (I.-T.L.); d93921032@gmail.com (T.-H.H.); liupoyu@gmail.com (P.-Y.L.)
† These authors contributed equally to this work.

Abstract: Treating type 2 diabetes (T2D) patients with sodium–glucose cotransporter 2 (SGLT2) inhibitors may be associated with an increased risk of urinary tract infections (UTIs), such as diabetes-induced asymptomatic bacteriuria. Pyuria—a condition wherein leukocytes are detected in the urine—is a predictor of UTIs. The aim of this study was to examine the urinary microbiome of Taiwanese T2D patients, with or without pyuria, undergoing SGLT2 treatment. We recruited seven T2D patients, recorded their clinical and biochemical characteristics, and collected their urine samples for 16S metagenomic sequencing. The primary outcomes were the diversity of urinary microbiota and the relative abundance of different species. We found that the microbiome of the pyuria group was significantly less diverse than the non-pyuria group (0.24 ± 0.04 vs. 2.21 ± 0.28, $p = 0.002$), while the number of operational taxonomic units did not differ significantly (763.5 ± 78.67 and 747 ± 141.3, $p = 0.92$). Escherichia-Shigella spp. dominated the microbiome of the pyuria group (97.4%–99.4%), and these patients tended to have more comorbidities. In conclusion, pyuria is associated with urinary microbiota dysbiosis in T2D patients being treated with SGLT2 inhibitors.

Keywords: type 2 diabetes; pyuria; bacteriuria; metagenomics; urinary tract infection; dysbiosis

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1. Introduction

Urinary tract infections (UTIs) are the most common infections among patients with type 2 diabetes (T2D), especially the complications associated with asymptomatic and symptomatic bacteriuria [1,2]. UTIs may impair kidney function and are associated with poor glycemic control and bad quality of life [3]. The development of novel antidiabetics further complicates the issue. For example, the use of sodium–glucose cotransporter 2 (SGLT2) inhibitors may be associated with a higher risk of UTIs [4,5].

SGLT2 inhibitors lower the blood glucose level by reducing its reabsorption. This creates a friendly environment for the growth of microorganisms due to SGLT2-inhibitor-induced glycosuria, eventually manifesting in a significantly higher risk of UTIs and

genital infections. According to a Food and Drug Administration report, 19 identified cases of urosepsis involved treatment with SGLT2 inhibitors [6]. Another case–control study reported that these drugs increase the risk of asymptomatic bacteriuria, a strong risk factor for developing subsequent recurrence in diabetic patients [7]. Patients treated with SGLT2 inhibitors may be five times more likely to contract genital mycotic infections, with the likelihood increasing in the first month before plateauing during the course of treatment [8].

A population-based study examined the association between SGLT2 inhibitors and the risk of serious adverse events including lower limb amputation and diabetic ketoacidosis [9]. In the EMPA-REG OUTCOME trial, it was reported that SGLT2 inhibitors could lead to serious urinary tract infections [10]. Lega and colleagues reported that SGLT2 inhibitors were associated with genital mycotic infections. However, the influence of SGLT2 inhibitors on the risk of UTIs was unclear in terms of urinary microbiome [11]. Elevated urine glucose levels induced by chronic hyperglycemia alter the microenvironment of the urinary tract, which might further alter the urinary microbiota. Liu et al. reported that urine specimens collected from females with T2D exhibited a lower diversity and richness of urinary microbiota [12].

Microscopic pyuria is widely accepted as a predictor of UTIs. It is a condition wherein one can observe, in a fresh unspun, unstained urine specimen, $\geq$5 leukocytes under a microscope at a high magnification [13]. However, in these cases, often, no bacteria are detected via conventional urine culture [1]. The dysbiosis of the urinary microbiome is a remarkable sign in the development of UTIs in specific populations such as kidney transplant patients [14]. Understanding the changes in the urinary microbiome will provide insights into the development of UTIs in diabetic patients undergoing SGLT2 therapy.

Accordingly, we speculate that an alteration of the urinary microbiota is involved in asymptomatic bacteriuria in diabetic patients. The goal of this study was to characterize the urinary microbiota in Taiwanese T2D patients by comparing the alpha and beta diversities as well as the specific genera involved and to use this information to shed light on the pathogenesis of diabetes-induced asymptomatic bacteriuria.

2. Materials and Methods

2.1. Patient Enrolment and Information

We enrolled adult patients with T2D with a recent history of using SGLT2 inhibitors between November 2010 and May 2020. The study was approved by the institutional review board of Taichung Veterans General Hospital (IRB no. CE21007A). We excluded patients who were currently menstruating, using antibiotics for more than 2 weeks, or had an indwelling catheter, all of which would likely interfere with the urinary microbiota. We collected the following patient information for analysis: baseline characteristics, including age, sex, and diabetic information; biochemical data, including glycosylated hemoglobin (HbA1c), total cholesterol (TC, mg/dL), triglycerides (TG, mg/dL), high-density lipoprotein cholesterol (HDL-C, mg/dL), low-density lipoprotein cholesterol (LDL-C, mg/dL), fasting plasma glucose (FPG, mg/dL), and urine albumin–creatinine ratio (UACR, mg/g); and medical chart records. Regarding definitions of DM and other complications, we adopted the criteria used by Khasriya et al. [13]. Mid-stream urine (50 mL) was collected, and 20 mL was used for standard cultivation for clinical examination. Pyuria was defined as the presence of $\geq$5 leukocytes/high-power field in a random urine sample [15], stratified according to the pyuria or non-pyuria group. The estimated glomerular filtration rate (eGFR) indicated the extent of kidney damage. eGFR stage II (60–89 mL/min) signified mildly impaired kidney function.

2.2. DNA Extraction, PCR, and MiSeq Sequencing

Within an hour, the resting specimens were transferred to the laboratory and centrifuged at 16,000$\times$ g for 10 min. Pellets were stored at -80 °C until further processing [16]. Total DNA was extracted from urine samples using the cetyltrimethylammonium bromide/sodium dodecyl sulfate method. DNA concentration and purity were monitored on

1% agarose gels, and the DNA was diluted to 1 ng/μL in sterile water. The V3–V4 regions of 16S rDNA were amplified via PCR using specific primers with barcodes. All the PCR reactions were carried out in a volume of 25 μL with the following composition: 0.5 μL of KAPA© High-Fidelity PCR Master Mix (KAPA BIOSYSTEMS, Wilmington, MA, USA), 0.5 μM of forward and reverse primers, and 1 ng of template DNA. The thermal cycling program was as follows: initial denaturation at 95 °C for 3 min; 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and elongation at 72 °C for 30 s; final extension at 72 °C for 5 min. An equal volume of 1× loading buffer (containing SYBR green) was mixed with each PCR product and the mixture was electrophoresed on 2% agarose gels. Samples showing one bright main band between 450 and 500 bp underwent further analysis. PCR products, mixed in equi-density ratios, were purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Sequencing libraries were generated using the TruSeq Nano DNA Library Prep Kit (Illumina, San Diego, CA, USA), following the manufacturer's recommendations with index codes added. The library quality was assessed using the Qubit@ 2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and the Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina MiSeq platform that generated 300 bp paired-end reads [15].

2.3. Bioinformatic Analyses

We used the Quantitative Insights Into Microbial Ecology (QIIME, version 1.9.0) software to generate a table of operational taxonomic units (OTUs) based on a default similarity level of 97%, determined from the 16S rDNA sequence data [17]. The alpha diversity was calculated using the mothur software (version 1.39.5) as indices in QIIME, including the following: observed species, Chao 1 index, abundance-based coverage estimators index, Shannon index, and Simpson index. The difference between alpha diversities was evaluated using the Mann–Whitney U test in the R software (version 4.03) [16].

2.4. Statistical Analysis

Continuous variables were expressed as means ± standard error of the mean. Categorical variables were expressed as numbers (percentages). The independent *t*-test was used to compare continuous variables, while the chi-squared test or Fisher's exact test was used to compare categorical variables. Statistical analyses were performed using the Statistical Package for Social Sciences (IBM SPSS version 22.0; International Business Machines Corp, New York, NY, USA). A two-tailed p value < 0.05 was considered statistically significant. The correlation between biochemical indicators and various microbes was determined based on the Spearman rank correlation coefficient, and the results were visualized as a heatmap in R (using "heatmap" command).

3. Results

3.1. Clinical Characteristics of All Patients

The biochemical characteristics of the pyuria (n = 3) and non-pyuria (n = 4) groups are shown in Table 1. All their urine samples, collected for bacterial culture, showed negative results. The mean age was 59 ± 5.7 years in the non-pyuria group and 59.7 ± 6.5 years in the pyuria group (p = 0.942). The proportion of females in the pyuria group was 100%, significantly higher than that in the non-pyuria group (25%). The proportion of patients in eGFR stage II was 25% in the non-pyuria group and 100% in the pyuria group (p = 0.047, Table 1). In addition to these characteristics, HbA1c, FPG, TC, TG, HDL-C, LDL-C, hypertension, insulin used, and UTI history did not differ significantly between the two groups (Table 1). The shared characteristic of chronic kidney disease was pyuria. Pyuria likely results from UTIs, which are correlated with a lower eGFR [18].

Table 1. Clinical characteristics of T2D patients treated with SGLT2 inhibitor.

	Without Pyuria (*n* = 4)	**Pyuria (*n* = 3)**	***p* Value**
Duration of diabetes (y)	9.75 ± 6.12	14.33 ± 5.89	0.614
Age (y)	59 ± 5.7	59.66 ± 6.5	0.942
Gender (female, *n*)	1	3	
Urine WBC > 1 cell (%)	0%	100%	0.008 **
eGFR (mL/min/1.73 m^2) stage II (*n*)	25%	100%	0.047 *
UACR > 30 mg/g (%)	0%	33.3%	0.212
HbA1c (%)	7.15 ± 0.2	6.7 ± 0.3	0.336
FPG (mg/dL)	141 ± 13.1	116.33 ± 7.8	0.174
TC (mg/dL)	189.55 ± 24.9	130.33 ± 4.4	0.097
TG (mg/dL)	137.25 ± 23.4	130.33 ± 4.4	0.097
HDL-C (mg/dL)	49.5 ± 3.42	43 ± 3.21	0.226
LDL-C (mg/dL)	122 ± 22.84	77 ± 7.37	0.142
Hypertension (%)	50%	66%	0.723
Insulin use (%)	0%	66%	0.062
Urinary tract infection history (%)	0%	66%	0.062

** $p < 0.01$; * $p < 0.05$. T2D, type 2 diabetes; SGLT2, sodium–glucose cotransporter 2; WBC, white blood cell; eGFR, estimated glomerular filtration rate; UACR, urine albumin–creatinine ratio; HbA1c, hemoglobin A1c; FPG, fasting plasma glucose; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

3.2. Urinary Microbiota Differed between the Pyuria and Non-Pyuria Groups Treated with SGLT2 Inhibitors

Patients' mid-stream urine samples were collected, and 16S metagenomic sequencing was performed. To model the urine bacterial microbiota, we first calculated the number of identified species using OTUs, and then their abundance using the Shannon species diversity index. Our working hypothesis was that changes in the urinary microbiota are involved in the pathogenesis of asymptomatic bacteriuria in these patients. We identified 652–996 OTUs in the non-pyuria group and 581–1028 in the pyuria group (Table 2). The Shannon species diversity indices ranged from 1.542 to 2.725 in the non-pyuria group and from 0.126 to 0.339 in the pyuria group (Table 2). The non-pyuria group had a significantly higher Shannon diversity index than the pyuria group (2.21 ± 0.28 vs. 0.24 ± 0.04, $p = 0.002$), but their OTUs were similar (763.5 ± 78.67 and 747 ± 141.3, $p = 0.92$) (Figure 1A,B). Both groups had similar species richness, while species diversity was significantly altered in the pyuria group.

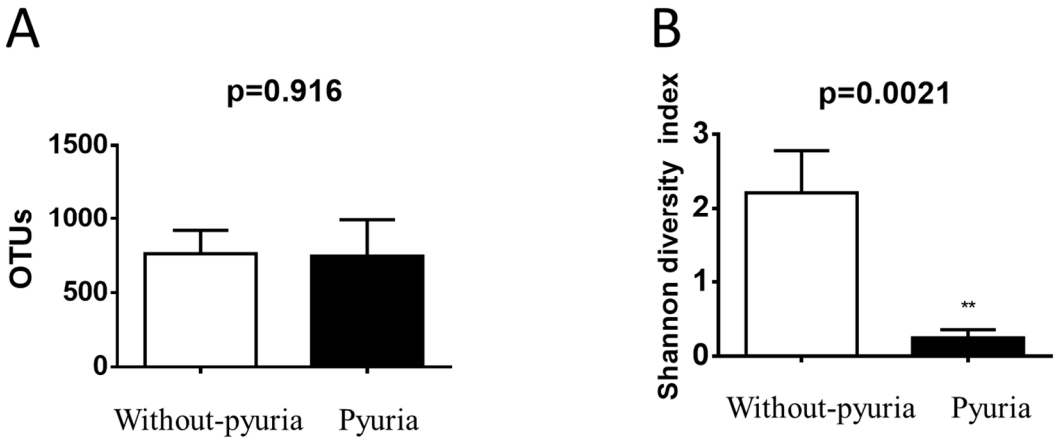

Figure 1. Comparison between the without-pyuria and pyuria groups for the number of OTUs (**A**) and the Shannon diversity index (**B**) in T2D patients treated with SGLT2 inhibitor. ** $p < 0.01$.

Table 2. Bacteria identified in the urine of SGLT2-inhibitor-treated T2D patients using 16S metagenomics.

Group	Sample ID	Shannon Diversity Index	Number of Species Identified (OTUs)	Top 5 Species				
				1	2	3	4	5
Without pyuria	Ur1	1.542	718	*Proteus mirabilis*	*Streptococcus agalactiae*	*Corynebacteriaceae*	*Escherichia-Shigella*	*Staphylococcus*
				47.08%	46.27%	1.87%	0.68%	0.65%
	Ur2	2.634	688	*Enterococcus faecalis*	*Corynebacterium*	*Veillonella*	*Bifidobacterium dentium*	*Escherichia-Shigella*
				45.20%	16.76%	13.46%	8.37%	6.07%
	Ur3	2.725	996	*Proteus mirabilis*	*Lactobacillus gasseri*	*Streptococcus anginosus*	*C. sp. NML 100378*	*Lactobacillus*
				33.46%	29.58%	16.29%	5.90%	4.77%
	Ur4	1.947	652	*Staphylococcus*	*Enterococcus faecalis*	*Corynebacteriaceae*	*Veillonella*	*kroppenstedtii*
				48.98%	30.39%	11.01%	5.49%	1.52%
Pyuria	Ur5	0.126	1028	*Escherichia-Shigella*	*Escherichia coli*	*Bifidobacterium dentium*	*C. pyruviciproducens ATCC BAA-1742*	*Staphylococcus*
				99.44%	0.14%	0.06%	0.05%	0.05%
	Ur6	0.339	632	*Escherichia-Shigella*	*Proteus mirabilis*	*Enterococcus faecalis*	*Streptococcus agalactiae*	*Staphylococcus*
				97.44%	0.63%	0.26%	0.24%	0.19%
	Ur7	0.276	581	*Escherichia-Shigella*	*Proteus mirabilis*	*Lactobacillus*	*Enterococcus faecalis*	*Streptococcus agalactiae*
				97.80%	0.42%	0.63%	0.18%	0.14%

OTU, operational taxonomic unit.

Interestingly, the 16S metagenomic sequencing data showed that *Escherichia-Shigella* spp. accounted for 97.4–99.4% of all species in the pyuria group (Figure 2, Table 2). *Proteus mirabilis*, *Enterococcus faecalis*, and *Staphylococcus* spp. were the three major species in the non-pyuria group (Table 2). These findings highlighted clear differences in the taxonomic composition of microbial communities from the two groups.

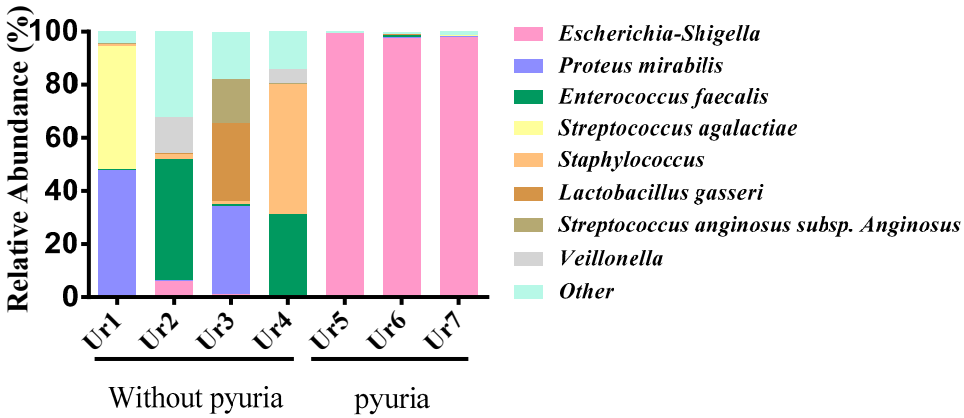

Figure 2. A stacked histogram showing the relative abundance of 16S metagenomic sequences in the urine of T2D patients treated with SGLT2 inhibitors.

3.3. Escherichia-Shigella Was Highly Correlated with Urine White Blood Cell (WBC) Count in Patients Treated with SGLT2 Inhibitors

To compare the urinary microbiota and clinical characteristics, we used Pearson correlation analysis. We found that Escherichia-Shigella was strongly and positively correlated with the urine WBC count ($p = 0.01$, Figure 3). These species were highly likely to be present in the urine of T2D patients treated with SGLT2 inhibitors. Koren et al. reported a positive correlation between levels of HDL and apolipoprotein A1, a major component of HDL [19]. Interestingly, we also found that Staphylococcus was positively correlated with HDL-C ($p = 0.03$, Figure 3). However, the mechanism underlying the correlation remains unclear. In addition, Streptococcus agalactiae border-line correlated with eGFR ($p = 0.05$) (Figure 3).

These observations suggest that urinary microbiota data can inform the early diagnosis and prognosis of T2D.

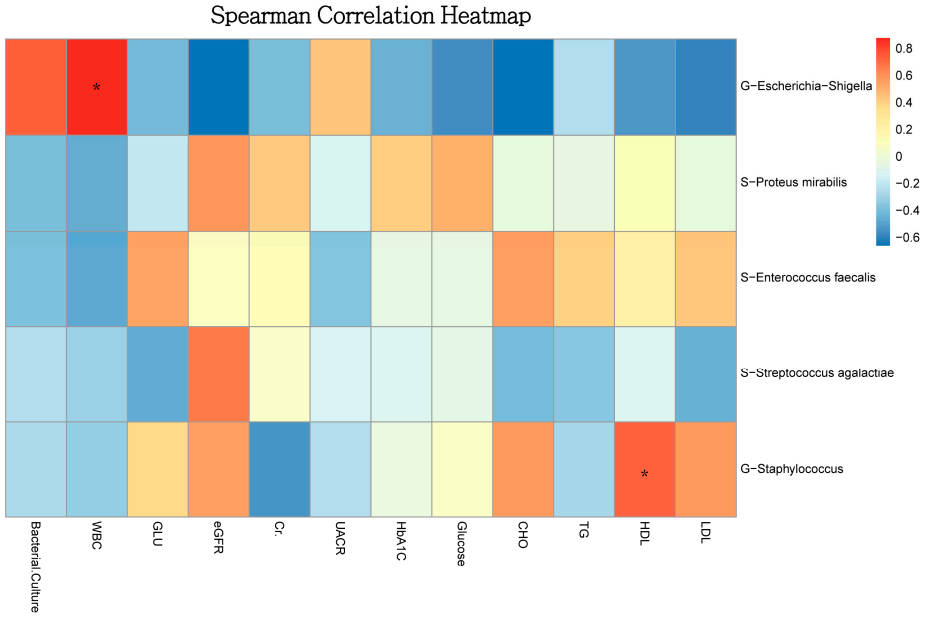

Figure 3. Pearson correlation coefficient heat map of mutual analysis between bacteria (genus or species) and clinical characteristics of 11 kinds. The magnitude of the R-value of the correlation analysis is displayed by color difference. * $p < 0.05$.

4. Discussion

In this study, we demonstrated, using 16S metagenomics, that the urine microbiota of T2D patients with pyuria majorly comprised Escherichia-Shigella spp., which could be associated with the development of UTIs in these patients. Females with T2D who have developed pyuria due to SGLT2 treatment have been shown to be more susceptible to microbial dysbiosis, particularly in the presence of excessive Escherichia-Shigella spp., compared with those without pyuria [7,20]. Yoo et al. also detected Escherichia-Shigella spp. using next-generation sequencing, whereas clinical urine cultures failed to do so for patients experiencing acute uncomplicated cystitis and recurrent cystitis [21]. The clinical urine culture method, typically, has a low sensitivity; the probability of positive findings using this method in patients with acute cystitis is only 60% [22].

SGLT2 inhibitors are a newer class of antidiabetic medications that reduce serum glucose levels by inhibiting its reabsorption in the proximal tubule [23]. By inhibiting the glomerular filtration of glucose from reabsorption, glucose is moved from the blood to the urine. High blood glucose levels are associated with a higher risk of UTIs and genital infections (especially in women) due to the conversion of blood sugar into urine sugar. SGLT2 inhibitors elevate the risk of asymptomatic bacteriuria, which is a strong risk factor for developing subsequent recurrent or severe clinical UTIs in diabetic patients (odds ratio 1.26, $p = 0.01$) [7,24]. However, no report has yet explored the relationship between the use of SGLT2 inhibitors and pyuria. The mechanisms by which SGLT2 inhibitors influence the urinary microbiota or specific urinary microbes remain to be elucidated. Lee et al. studied diabetic mice treated with dapagliflozin, and they found microbiota richness, reduced diversity, and a low *Firmicutes/Bacteroidetes* ratio [25,26]. In line with that, we found that the composition of urinary microbiota in the pyuria group was markedly different from that in the non-pyuria group (Table 2, Figure 2).

Our findings are consistent with those of Chen et al., who reported that female T2D patients experienced serious lower urinary tract symptoms [16]. Consistent with Chen et al. and Tao et al., we found that the use of SGLT2 inhibitors in the pyuria group was associated

with the predominance of *Escherichia-Shigella* in their urine microbiota [16,20]. Further study is needed to identify specific dysbioses that might exacerbate diabetes-associated UTIs.

A recent study also found that female T2D patients with pyuria had higher incidences of diabetic retinopathy, neuropathy, nephropathy, cerebrovascular disease, and hyperlipidemia, compared with those without pyuria [15]. In summary, we have demonstrated, in diabetic patients treated with SGLT2 inhibitors, the association between pyuria and urine microbiome dysbiosis, together with other adverse outcomes. Future studies should identify the dynamic changes in microbiota underlying the observed association.

5. Limitations

There are several limitations in our study. First, T2D patients with suspicions of UTIs symptoms will be treated with a therapy through oral antibiotics administration before the results of bacterial cultures are obtained. However, it is not possible and it is even unethical to1112 postpone antibiotic administration after for the bacterial culture report. T2D patients' treatments with antibiotics may change their microbiomes. Secondly, the microbiome from only seven urine samples was analyzed; this these may not be properly representative of the urinary microbiome of T2D patients, with or without pyuria. Nevertheless, this is the first study to investigate treatment with SGLT2 inhibitors in patients with and without pyuria microbiomes with the utilization of the NGS sequencing method.

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Institutional Review Board Statement: This study was approved by the Institutional Review Board(I) of Taichung Veterans General Hospital (protocol code CE21007A).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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