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Effect of SGLT2 Inhibitors + DPP-4 Inhibitors on Urine Microbiota in Type 2 Diabetes

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ABSTRACT

Aims: A reduced compliance, due to urogenital minor infections, frequently compromises the clinical efficacy of SGLT2 inhibitors in subjects with type 2 diabetes (T2D). The combined use of SGLT2 inhibitors and dipeptidyl-peptidase four inhibitors seems to reduce the incidence of such side effects. We evaluated how these drugs, alone or in combination, might influence resident urinary microbiota.

Materials and Methods: An open label, randomised clinical study was conducted on 30 T2D individuals for 12 weeks to compare the impact of Empagliflozin and Empagliflozin/Linagliptin on clinical parameters and urinary microbiota. Fifteen healthy individuals served as baseline controls. The composition of urinary bacterial populations was evaluated by Real-Time quantitative PCR and 16S rRNA gene sequencing.

Results: BMI was reduced by both treatments, while fasting glucose and HbA1c significantly improved only with the combination. At baseline, T2D showed a higher total bacterial load and abundance of *Bacillota* than controls. The prevalence and proportion of bacterial species profoundly differed between the groups, revealing a urinary dysbiosis in T2D. A different effect of Empagliflozin alone or combined with Linagliptin on microbial populations was observed: Empagliflozin increased the total bacterial load of *Bacillota* and *Aerococcus*, while the combination therapy restored a microbial community similar to that of controls, further reducing the prevalence of potential urinary pathogens.

Conclusions: In T2D subjects, the combination of Empagliflozin and Ligandliptin might help in restoring a normal composition of the urinary microbiota, likely improving compliance and persistence in therapy with SGLT2 inhibitors.

1 | Introduction

The need for implementing life-saving therapy as soon as possible is becoming urgent for chronic, non-communicable diseases. In addition to their effective glucose-lowering action, in the last 10 years increasing evidence from randomised clinical trials and real-world studies has supported SGLT2 inhibitors (SGLT2i) as key therapy in preventing and slowing the progression of chronic kidney disease and heart failure in subjects with and without type 2 diabetes (T2D) [1–4]. Therefore, SGLT2i evolved from glucose-lowering drugs to essential therapeutic options to improve the prognosis of the cardio-renal-metabolic (CRM) syndrome [5], and the population that would benefit from SGLT2i therapy is wide-ranging. However, the full efficacy of these drugs strictly depends on their persistence overtime in the patient's therapeutic scheme. Several causes might compromise such issue, from therapeutic inertia by clinicians [6, 7], who may erroneously believe that they are unable to effectively treat CRM syndrome, to concerns for drug-related adverse events outweighing the benefits [8], and poor

adherence to treatment by patients, with high discontinuation rates [9, 10].

SGLT2i are not burdened by major side effects, but the undisputable increased frequency of low tract genitourinary infections, reported as affecting 4%–5% of participants in randomised clinical trials, is higher in the clinical practice [11], thus configuring a barrier against the persistence in therapy. Interestingly, combining SGLT2i with dipeptidyl-peptidase four inhibitors (DPP-4i) has been shown to lower the incidence of both urinary and genital tract infections compared to using SGLT2i alone [12], making it a preferable option for patients concerned about infections. Putative mechanisms behind such protection still deserve investigation.

As for other microbial communities residing in humans, the composition, dynamics, and functions of the lower urinary tract microbiota have profound clinical implications. Its imbalance is considered a potential cause of functional disorders such as overactive bladder, urinary incontinence, interstitial cystitis, and chronic prostatitis [13, 14], with specific bacterial communities associated with these disorders.

Individuals with T2D show frequent significant changes in the urinary microbiota, such as a higher total bacterial load and increased abundance of certain bacterial groups, such as *Bacillota*, compared to healthy subjects [15]; however, available studies were performed in females, mainly from China [16, 17], and information on male T2D subjects is lacking.

The present study aimed to compare the effect of Empagliflozin, a SGLT2i, alone or in combination with Linagliptin, a DPP-4i, on the resident microbiota of the lower urinary tract in subjects with T2D, in the attempt to define mechanisms explaining why the combination therapy SGLT2i + DPP-4i is reported to be better tolerated than monotherapy with SGLT2i.

2 | Subjects and Methods

Participants and study design 30 subjects with T2D were recruited in the outpatient diabetes clinic of the Santa Chiara University Hospital in Pisa, Italy. Inclusion criteria were age 50–80 years, HbA1c < 8% (64 mmol/mol), clinical indication for treatment with SGLT2i, preserved kidney function (eGFR > 60 mL/min/1.73 m^2), no previous major cardiovascular events. Exclusion criteria were antimicrobial therapy (ongoing or in the last 4 weeks); previous use of SGLT2i, GLP-1 RA, or DPP-4i; infective, irritative, and/or obstructive genitourinary symptoms; anatomical or functional abnormalities of the urinary tract. In addition, 15 healthy subjects, matched by age and gender to the 30 T2D subjects and adhering to the inclusion/exclusion criteria, were included in the study as controls. Prior to recruitment, subjects were informed of the purpose of the study and expressed their consent to participation and withdrawal by registration in accordance with the Declaration of Helsinki. The study was carried out in accordance with the principles of Declaration of Helsinki and approved by the Ethics Committee of the Tuscany Region—North-West Vast Area (CEAVNO, protocol no. 17606). The study was registered on clinicaltrials.gov (NCT04735042). Figure S1: Figure S1 shows the flow diagram of participant recruitment, randomisation, and retention.

At time 0 (T_0), after an overnight fasting, vital parameters were recorded and blood samples were drawn from antecubital veins for routine analyses, measured on plasma or serum aliquots using standard techniques. Morning mid-late-stream urine samples were collected from all participants in sterile containers for routine urine culture analysis. Urine samples were immediately tested for genitourinary infections using the standard culture procedure by streaking urines on blood agar and MacConkey agar plates (Meus, Italy) and incubating plates at 37°C for 48 h.

The following day, T2D subjects were randomised in a 1:1 ratio to receive Empagliflozin (10 mg/day) or Empagliflozin/Linagliptin (10 mg/day + 5 mg/day) as add-on to previous ongoing therapy. Group allocation was established using computer-generated random numbers. Personnel performing laboratory measurements were blinded to group allocation. Phone questionnaires were performed every 4 weeks to collect information regarding the tolerability of the treatment and the occurrence of any side effect. Subjects were advised to refrain for changing any ongoing chronic pharmacologic treatment, like anti-hypertensive or hypolipidemic drugs, for the whole duration of the study. Compliance to treatments was ascertained by monthly checking pills count. After 12 weeks (T_1), clinical parameters were recorded and blood and urine samples collected as at T_0. This study duration was expected to ensure the clinical effect of the drug on HbA1c and body weight [18].

Biochemistry Biochemical analyses were performed at the clinical laboratory of the University Hospital of Pisa. Plasma glucose was measured by an enzymatic method using glucose oxidase and peroxidase on an automated analyser (Hitachi 912, Roche, Switzerland). HbA1c was measured by DCCT assay in standardised high-performance liquid chromatography. Total and high-density lipoprotein cholesterol, and triglycerides were measured by routine methods; low-density lipoprotein cholesterol was calculated by the Friedwald formula. Serum creatinine was measured by the modified Jaffe method and estimated glomerular filtration rate was calculated from the creatinine-based 2021 CKD-EPI equation [19].

DNA Extraction Immediately after urine cultures were performed, 50 mL of urine was transferred to sterile tubes and centrifuged at 4500 rpm for 10 min at 4°C. Supernatants were removed, and pellets were washed with 10 mL of sterile phosphate buffered saline (PBS; 1 M KH_2PO_4, 1 M K_2HPO_4, 5 M NaCl, pH 7.2) by centrifuging at 4500 rpm for 10 min at 4°C. After removing supernatants, pellets were stocked at −80°C until use.

Microbial genomic DNA was extracted from urinary sediments using the QIAamp DNA Mini Kit (QIAGEN, Germany) according to the manufacturer's protocol. The quality and quantity of DNA was checked using the NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, USA).

Absolute Quantification of Bacterial Populations Absolute abundances of the total bacterial load and the main phyla

(i.e., *Actinomycetota*, *Bacillota*, *Bacteroidota*, *Pseudomonadota*) and genera (i.e., *Aerococcus*, *Corynebacterium*, *Gardnerella*, *Lactobacillus*, *Prevotella*, *Streptococcus*) constituting the urinary microbiota were assessed on extracted DNAs by 16S rRNA gene-targeting Real-Time quantitative polymerase chain reactions (qPCRs), using primer pairs targeting phylum- or genus-specific 16S rDNA regions (Supporting Information S1: Table S1). A primer pair targeting a sequence of the 16S rRNA gene conserved in all bacteria was used for the quantification of the total bacterial load (Table S1). qPCRs were performed using the CFX96 Real-Time System (BioRad, USA) and CFX Maestro Software (version 2.3, BioRad). All reactions were carried out in duplicate in a 96-well plate with a final volume of 20 μL per well, including 8 μL of sterile water, 0.5 μL of each primer (10 μM), 10 μL of Luna Universal qPCR Master Mix (New England BioLabs, USA), and 1 μL of template DNA (normalised to a standard concentration of 2 ng DNA/μL). The amplification protocol comprised: an initial denaturation step at 95°C for 1 min followed by 45 cycles of denaturation at 95°C for 15 s, annealing at each primer set specific temperature (Table S1) for 30 s, and extension at 72°C for 10 s. Absolute quantifications were performed by comparing with calibration curves generated using external standards with known concentrations ranging from 10^2 to 10^{10} DNA copies/μL.

SixteenS rRNA Gene Sequencing and Metagenomic Analysis 16S rRNA gene sequencing and data processing were performed by Novogene (Beijing, China). V3–V4 16S rRNA gene hypervariable regions were amplified with primers 341F and 806R. PCR products were purified from a 2% agarose gel with the QIAGEN Gel Extraction Kit (QIAGEN). Sequencing libraries were generated using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England BioLabs), and their quality was evaluated by the Qubit@2.0 Fluorometer (Thermo Fisher Scientific) and the BioAnalyzer 2100 System (Agilent Technologies, USA). Libraries were sequenced on the HiSeq Illumina platform, and pair-ended 250 bp reads were generated. Raw data were filtered using QIIME2, and high-quality reads (55–132K reads per sample obtained after filtering) normalised by rarefaction to 30K reads per sample. Reads were clustered in operational taxonomic units (OTUs) with a ≥ 97% similarity cut-off using Uparse. Representative sequences of each OTU were analysed using the RDP classifier for taxonomic resolution. Phylogenetic relations between OTUs were assessed with MUSCLE, and α-diversity analysis was performed using QIIME2 and *R*.

Antimicrobial Activity of Empagliflozin and Linagliptin Tablets of Empagliflozin and Linagliptin were dissolved in sterile water by thoroughly vortexing to a final concentration of 4 mg/mL each. Empagliflozin/Linagliptin was obtained by mixing the two drug suspensions in a ratio 2:1, following the proportion of the daily administration of the combination therapy to T2D subjects. Antimicrobial susceptibility testing was performed by the broth microdilution method, according to EUCAST guidelines for bacteria and fungi. *Acinetobacter baumannii* ATCC 19606, *Bacillus cereus* ATCC 14579, *Candida albicans* ATCC 10231, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Listeria monocytogenes* ATCC 13932, *Pseudomonas aeruginosa* ATCC 15442, and *Staphylococcus aureus* ATCC 12600 were used. Microbial suspensions were prepared in cation-adjusted Mueller-Hinton broth (for bacteria) or RPMI 1640 + 2% glucose (for fungi) starting from isolated colonies to $OD_{600} \approx 0.1$ (corresponding to $\approx 1.5 \times 10^8$ CFU/mL for bacteria and $\approx 1 \times 10^6$ CFU/mL for yeasts). 100 μL of the microbial suspensions were inoculated in 96-well polystyrene microplates containing scalar (2-folds) concentrations from 0.0625 to 1 mg/mL for Empagliflozin and Linagliptin and from 0.0625/0.0312 to 1/0.5 mg/mL for Empagliflozin/Linagliptin. Microplates were incubated at 37°C for 24 h, and the minimal inhibitory concentrations (MICs) determined.

Statistics Data are expressed as a mean ± standard deviation, setting statistical significance to a p-value < 0.05. Student's t-test for unpaired data was applied to compare the results of clinical biochemistry, Real-Time PCR, and sequencing between healthy controls and T2D subjects. Student's t-test for paired data was applied to compare the composition of the urinary microbiota at T_0 (pre-treatment) and T_1 (post-treatment). One-way ANOVA followed by Dunnet's test was used to compare Real-Time PCR and sequencing data by groups and treatments. Fisher's test was applied to infer significant differences in the prevalence of bacterial species between controls and T2D subjects and between T_0 and T_1. Sequencing data were also analysed through a Principal Component Analysis (PCA). Statistical analyses were performed using GraphPad Prism software (version 9.5.0, Dotmatics, USA).

3 | Results

All recruited T2D subjects completed the study and were included in the analysis. Clinical characteristics of the study participants at baseline are shown in Table S2. Male subjects prevailed. Controls and T2D subjects were comparable for age, BMI, and lipid profile. Blood pressure values were significantly higher in T2D. 12 men (10 metformin, 2 insulin) and 9 women (7 metformin, 2 insulin) were pharmacologically treated for T2D.

Table 1 shows the effect of 12 weeks of therapy with Empagliflozin or Empagliflozin/Linagliptin on clinical and biochemical parameters in these subjects. Both groups displayed a significant reduction of BMI, while fasting glucose and HbA1c significantly improved only with the combination. No differences in blood pressure or lipid profile emerged between the two treatments.

Baseline urinary microbiota in T2D To verify the composition of microbial communities in the lower urinary tract, genomic DNA extracted from urinary sediments was analysed using a combined approach of Real-Time PCR and sequencing, both having as molecular target the gene encoding the bacterial 16S rRNA. Results show that, at baseline, the total bacterial load ($p = 0.0473$) and levels of *Bacillota* ($p = 0.0463$) were higher in T2D than in controls (Figure 1A); a trend toward a difference between the two groups, although not statistically significant, was also observed for phyla *Actinomycetota* ($p = 0.0596$) and *Bacteroidota* ($p = 0.1780$) (Figure 1A), suggesting a possible involvement of the main phyla of the urinary microbiota in the increase of total bacterial load. No quantitative differences in the tested genera were observed (Figure 1B). Relative

TABLE 1 | Effect of 12 weeks of treatment with Empagliflozin or Empagliflozin/Linagliptin on clinical and biochemical parameters.

	Empagliflozin ($n = 15$)			Empagliflozin/linagliptin ($n = 15$)		
	T_0	T_1	p	T_0	T_1	p
Age (yrs)	63.1 ± 9.4		—	67.4 ± 8.9		—
Diabetes duration (yrs)	4.2 ± 0.6		—	4.0 ± 0.8		—
BMI (kg/m^2)	28.1 ± 1.5	26.8 ± 1.2	0.023	29.2 ± 1.0	28.2 ± 1.1	0.034
SBP (mmHg)	139 ± 4	136 ± 5		147 ± 3	142 ± 5	
DBP (mmHg)	80 ± 3	81 ± 3		83 ± 2	83 ± 3	
Plasma glucose (mg/dL)	139 ± 9	130 ± 8		142 ± 9	115 ± 5	0.027
HbA1c (%)	7.0 ± 0.2	6.7 ± 0.2		7.5 ± 0.3	6.5 ± 0.1	0.002
Creatinine (mg/dL)	0.94 ± 0.06	0.97 ± 0.07		0.86 ± 0.04	0.95 ± 0.12	
eGFR (mL/min/1.73 m^2)	75.2 ± 5.1	82.9 ± 5.6		81.6 ± 5.0	82.8 ± 5.8	
Total cholesterol (mg/dL)	163 ± 11	159 ± 10		152 ± 9	153 ± 9	
HDL cholesterol (mg/dL)	45 ± 3	47 ± 3		51 ± 4	52 ± 4	
LDL cholesterol (mg/dL)	90 ± 9	87 ± 10		77 ± 7	80 ± 8	
Triglycerides (mg/dL)	140 ± 19	124 ± 18		108 ± 9	103 ± 10	

abundances obtained from the metagenomic analysis provided a wider overview of the composition of the urinary microbial consortia and confirmed the results obtained by qPCR for phyla (Figure 1C,D), with *Bacillota* representing the 9% of the consortium in controls and 27% in T2D ($p < 0.0001$); accordingly, a contextual relative reduction of *Actinomycetota* and *Pseudomonadota* was observed. The 16S rRNA gene sequencing showed that the α-diversity indices Chao1 ($p = 0.0568$) and Shannon ($p = 0.3091$) and the number of observed species ($p = 0.2359$) did not differ between controls and T2D subjects (Figure 1E), evidencing a similar biodiversity of bacterial populations in the two study groups.

Table S3 shows prevalences of the most abundant species identified in the study population. The prevalence of *Actinotignum urinale, Aerococcus christensenii, Anaerococcus hydrogenalis, Campylobacter ureolyticus, Corynebacterium aurimucosum, Corynebacterium coyleae, Corynebacterium glucuronolyticum, Enterococcus faecalis, Escherichia coli, Ezakiella massiliensis, Gardnerella vaginalis, Lactobacillus iners, Mobiluncus curtisii, Peptoniphilus grossensis, Prevotella buccalis, Veillonella atypica,* and *Veillonella montpelliensis* was increased in concomitance with T2D, while that of *Actinotignum schalii, Bifidobacterium breve, Corynebacterium pyruviciproducens, Facklamia hominis, Lactobacillus gasseri, Peptoniphilus lacrimalis, Schaalia radingae,* and *Streptococcus agalactiae* was significantly reduced (Table S3).

Effect of Empagliflozin and Empagliflozin/Linagliptin on the urinary microbiota Real-Time PCR data pointed out a different effect of Empagliflozin alone or combined with Linagliptin on microbial populations of the lower urinary tract (Figure 2A). Total bacterial load ($p = 0.0006$) and abundances of *Bacillota* ($p = 0.0052$) and *Aerococcus* ($p = 0.0084$) were increased by Empagliflozin when compared with healthy controls; Empagliflozin also induced a significant reduction of *Prevotella* ($p = 0.0348$). Combination therapy administered to T2D individuals was apparently able to solve the urinary dysbiosis and partially restore the microbial abundances typical of healthy subjects, since not significant differences from healthy control were observed, except for *Prevotella* ($p = 0.0461$) (Figure 2A). When comparing T_0 versus T_1 in T2D individuals, no significant difference emerged for the tested taxa based on the treatment (Figure 2B).

The Chao1 ($p = 0.9833$) and Shannon ($p = 0.7512$) α-diversity indices and the number of observed species ($p = 0.9932$) did not differ between the two treatments (Figure 3A). PCA showed that samples from healthy controls and T2D individuals clustered in two separate groups ($p < 0.0001$), while overlapping clusters with no significant differences were evidenced for Empagliflozin and Empagliflozin/Linagliptin (E vs. E/L: $p = 0.7896$) (Figure 3B). However, both treatments resulted significantly different from healthy controls (E: $p = 0.0051$; E/L: $p = 0.0004$) but not from T2D (E: $p = 0.5730$; E/L: $p = 0.8624$) (Figure 3B). In addition, results obtained from the 16S rRNA-gene DNA sequencing confirmed Real-Time PCR data regarding bacterial phyla, although providing further insights and highlighting novel differences for the main bacterial genera (Figure 3C,D) and species (Table S4). Among over 90 analysed species, reported in Table S4, the prevalence of only 5 of them significantly varied after the treatments compared to baseline (Table 2); in detail, *Actinomyces urogenitalis* ($p = 0.0421$), *Campylobacter ureolyticus* ($p = 0.0169$), *Peptoniphilus coxii* ($p = 0.0421$), *Porphyromonas bennonis* ($p = 0.0352$), and *Prevotella bergensis* ($p = 0.0421$) resulted less prevalent versus T_0 after Empagliflozin/Linagliptin, while prevalence of *Porphyromonas bennonis* was reduced only in T2D subjects receiving Empagliflozin ($p = 0.0421$).

Lastly, to define if the two molecules displayed any direct antimicrobial activities on microbial communities of the lower urinary tract, antimicrobial susceptibility testing with Empagliflozin, Linagliptin, and Empagliflozin/Linagliptin was performed. Obtained Minimum Inhibitory Concentration (MIC) values for each strain are reported in Table S5. Empagliflozin and Linagliptin alone and their combination displayed

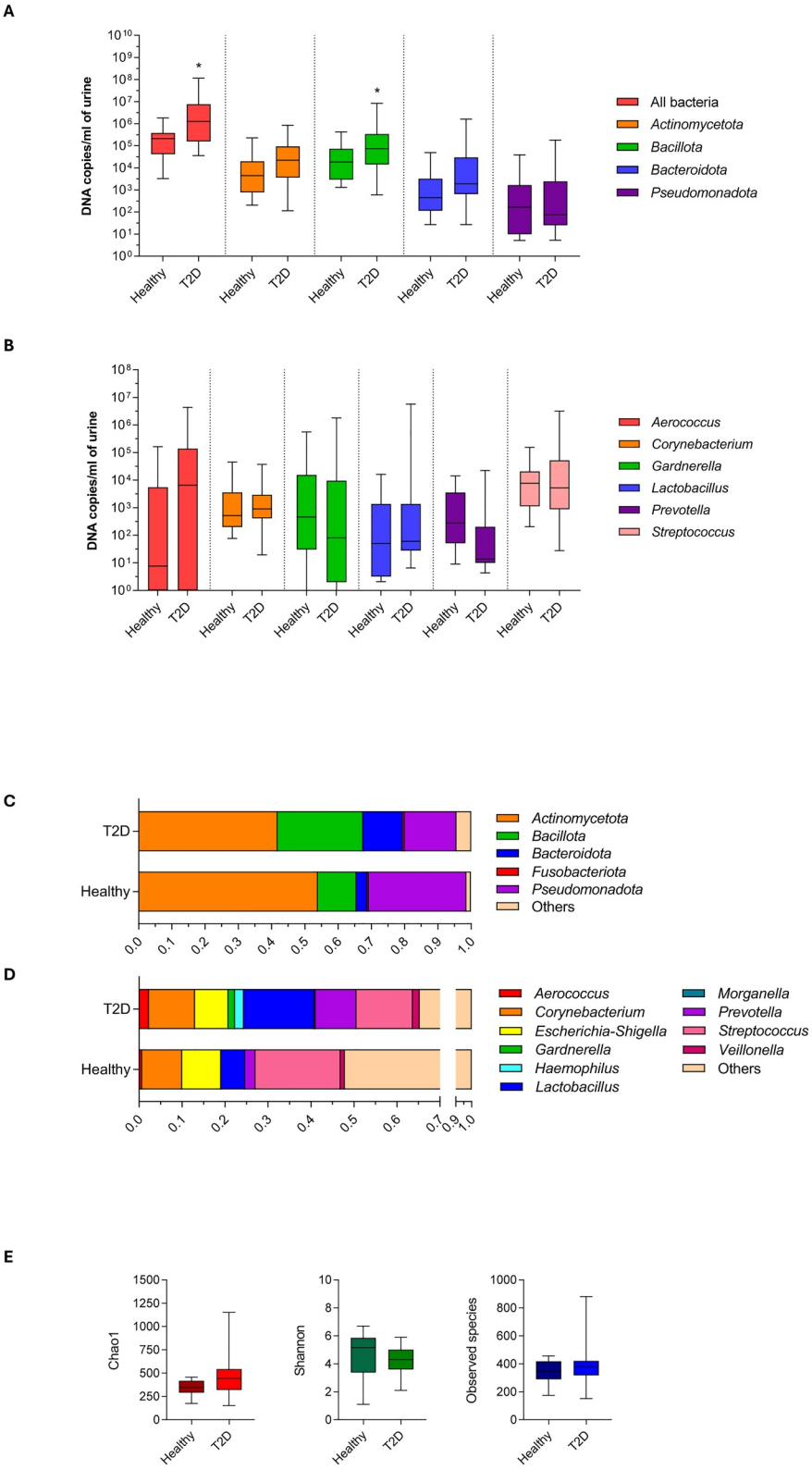

FIGURE 1 | Composition of the urinary microbiota in healthy controls and individuals with type 2 diabetes (T2D). Absolute abundances (DNA copies/mL of urine) of total bacterial load and main bacterial phyla (A) and main bacterial genera (B) constituting the urinary microbiota obtained by Real-Time PCR. Relative abundances of main bacterial phyla (C) and main bacterial genera (D) constituting the urinary microbiota obtained by 16S rRNA gene sequencing. α-diversity indexes (Chao-1 and Shannon) and observed species obtained by 16S rRNA gene sequencing (E).

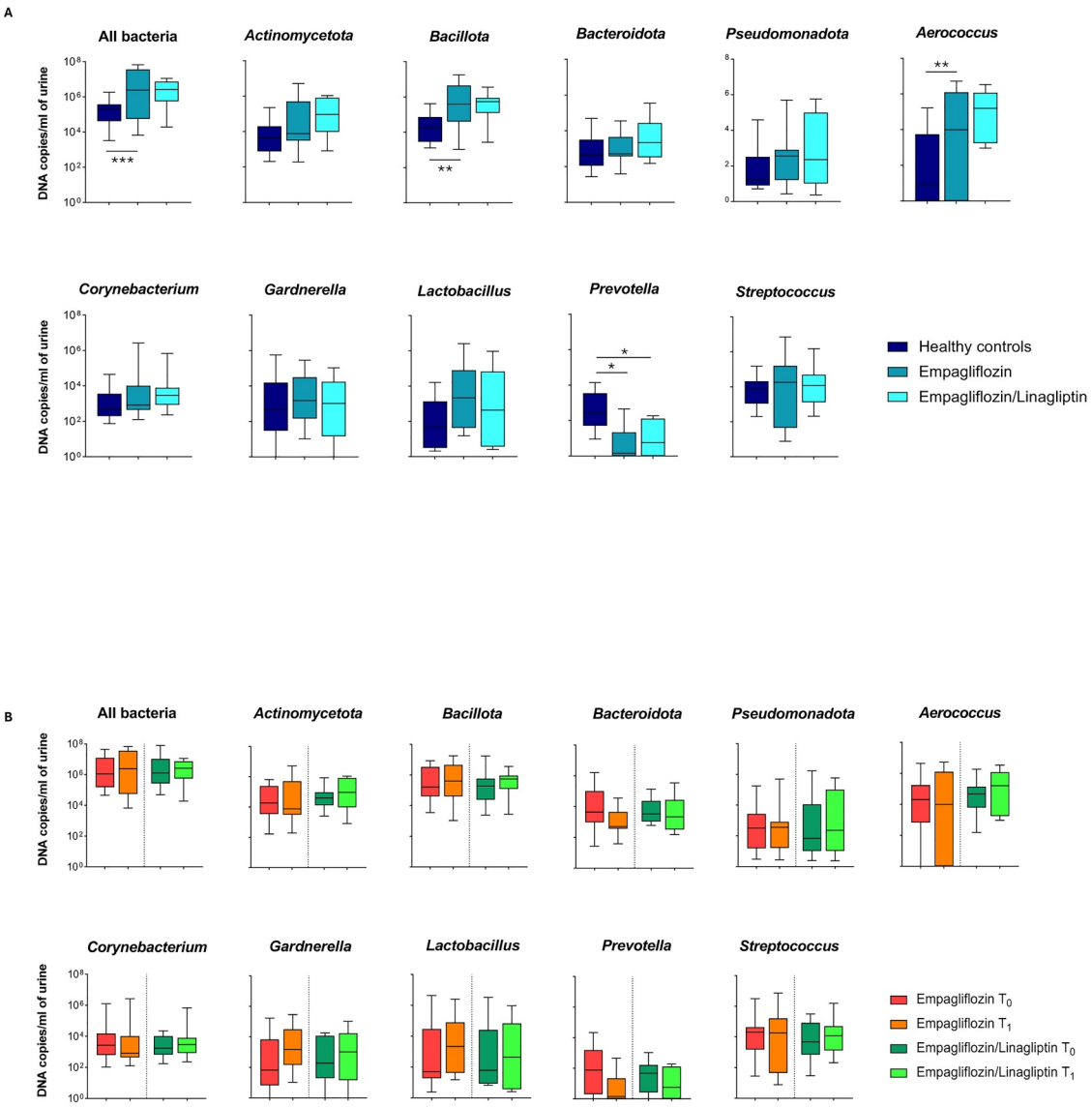

FIGURE 2 | (A) Effects of Empagliflozin and Empagliflozin/Linagliptin on the urinary microbiota in T2D individuals. Data are compared with those obtained in healthy controls. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. (B) Effects of Empagliflozin and Empagliflozin/Linagliptin on the urinary microbiota in T2D individuals before (T_0) and after (T_1) the treatment.

antimicrobial activity in concentrations generally higher than 0.25 mg/mL against Gram-positive and Gram-negative bacteria, as well as yeasts. These findings confirm in vitro antimicrobial activities of Empagliflozin and Linagliptin, alone or in combination.

4 | Discussion

The present study investigated the impact of treatment with Empagliflozin, alone or in combination with Linagliptin, on the composition of the urinary microbiota in T2D individuals. The results show that Empagliflozin monotherapy is associated with a significant modification of the urinary microbiota, characterised by the increase of specific bacterial taxa, while the combination Empagliflozin/Linagliptin preserves a microbial profile more similar to that observed in healthy controls.

These observations are part of the growing interest in the modulation of gut and urinary microbiota as key element in the pathogenesis of different complications associated with T2D [20, 21], and in the mode of action of SGLT2i, with studies so far conducted almost exclusively in animal models [22, 23]. Previous evidence already documented an increased risk of urinary tract infections in subjects with and without T2D treated with this class of drugs [24, 25], although the real-life clinical incidence is variable. The higher incidence of infections positively correlates with the higher glycosuria promoted by SGLT2i, suggesting that urine enriched in glucose represents a more favourable environment for some microorganisms, including pathogens, to replicate and determine infections.

We show here for the first time as SGLT2i can modify the urinary microbial communities, presumably by increasing the availability of glucose. Furthermore, our study provides another important novelty: the worsening of dysbiosis induced by Empagliflozin in

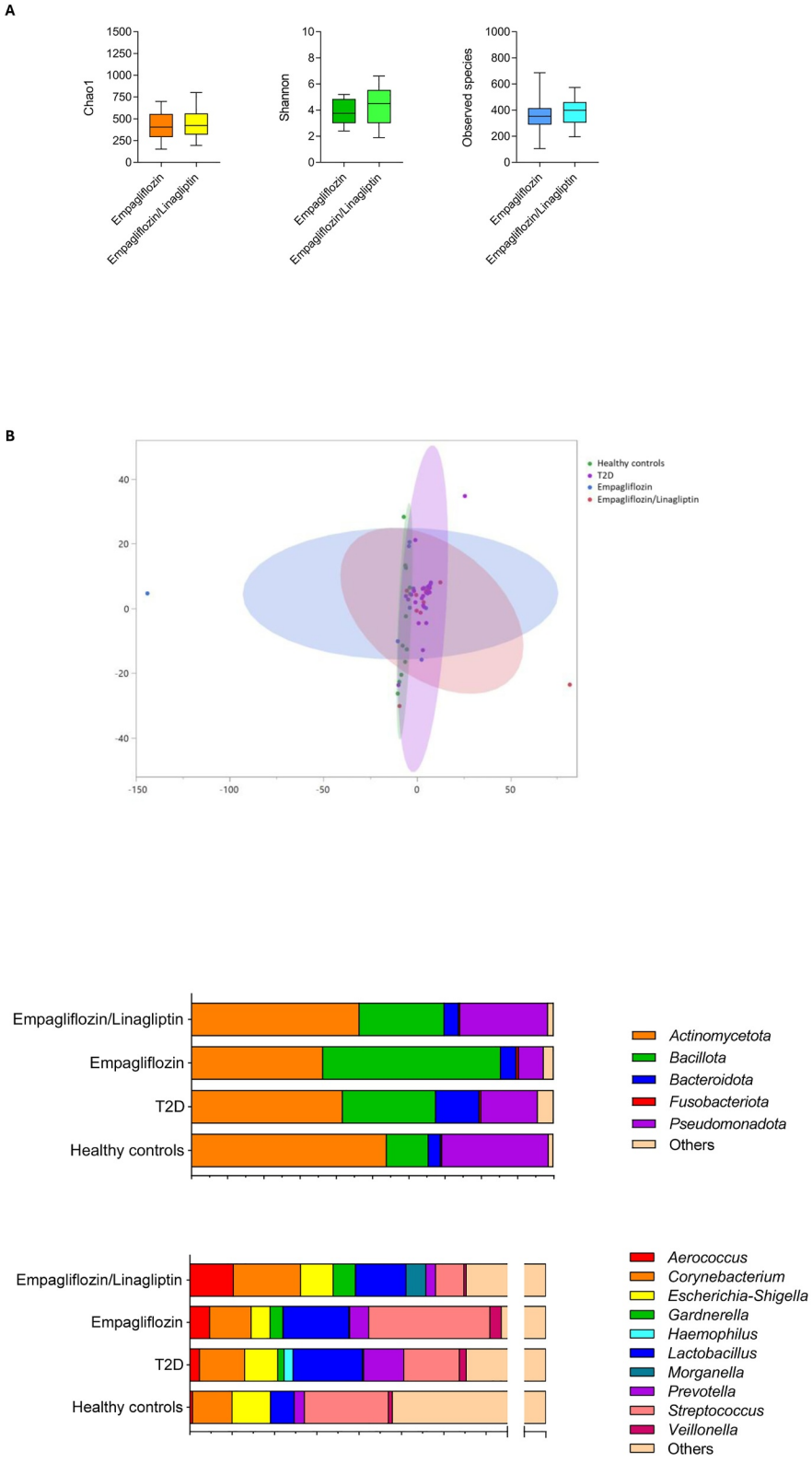

FIGURE 3 | Effects of Empagliflozin and Empagliflozin/Linagliptin on the urinary microbiota in T2D individuals according to 16S rRNA gene sequencing. (A) α-diversity indexes (Chao-1 and Shannon) and observed species. (B) Principal Component Analysis plot. (C) Relative abundances of the main bacterial phyla. (D) Relative abundances of the main bacterial genera.

T2D subjects can be counteracted by the co-administration of a DPP-4i, which might potentially translate into a clinical advantage. While Empagliflozin alone maintained, or even worsened, the urinary dysbiosis occurring in concomitance with T2D (i.e., increased total load, *Bacillota*, *Aerococcus*), the combination of drugs restored a healthier microbial composition similar to that of control individuals. In addition, Empagliflozin/Linagliptin, but not Empagliflozin alone, was found to reduce the prevalence of

TABLE 2 | Prevalence of the main bacterial species that significantly differed before and after the treatment with Empagliflozin or Empagliflozin/Linagliptin in T2D individuals.

Bacterial species	Empagliflozin T_0		Empagliflozin T_1		Significance	Empagliflozin/linagliptin T_0		Empagliflozin /linagliptin T_1		Significance	
	Out of 15	%	Out of 15	%	*p*-value	Out of 15	%	Out of 15	%	*p*-value	
Actinomyces urogenitalis	3	20	0	0	0.2241	5	33.3	0	0	0.0421	*
Campylobacter ureolyticus	12	80	8	53.3	0.2451	15	100	9	60	0.0169	*
Peptoniphilus coxii	2	13.3	0	0	0.4828	5	33.3	0	0	0.0421	*
Porphyromonas bennonis	5	33.3	0	0	0.0421	7	46.7	1	6.7	0.0352	*
Prevotella bergensis	1	6.7	0	0	> 0.9999	5	33.3	0	0	0.0421	*

*$p < 0.05$.

potentially pathogenic species, including *Actinomyces urogenitalis*, *Campylobacter ureolyticus*, *Peptoniphilus coxii*, *Porphyromonas bennonis*, and *Prevotella bergensis* [26–30]. This lowering may contribute to the reduced risk of urinary infections showed by the combination therapy in the clinical practice [12]. However, it is still difficult to point out the biological meaning for some of the observed variations in microbiota composition, especially in largely uncharacterised microbial communities such as the urinary microbiota.

There are many possible mechanisms by which Linagliptin could exert this protective effect. It has been hypothesised that DPP-4i can modulate systemic and local inflammation, reducing the epithelial permeability and cell proliferation and affecting the secretion of antimicrobial peptides [31–33]. Of note, Linagliptin exhibited anti-inflammatory effects regardless the glycaemic control in preclinical and clinical settings [34–36]. Direct studies on the specific impact of GLP-1 receptor agonists or DPP-4i on urothelial mucosa or bladder contractility are not as readily available; we may just speculate that the regulation of the incretin axis could have an impact on the immune tone of the urothelial mucosa. From a clinical viewpoint, maintaining a urinary microbiota similar to the physiological one, as the Empagliflozin/Linagliptin combination did in this study, could translate into a lower incidence of infections, improving the safety profile of SGLT2i. It could be also hypothesised that the combination of the two molecules improves glucose control more than SGLT2i alone, thereby contributing to protection towards infections; in fact, in our study group, the combination significantly reduced both fasting glucose and HbA1c. This is particularly relevant considering that complicated genitourinary infections can worsen glycaemic control, increase the risk of hospitalisations, and have a negative impact on the quality of life of T2D subjects.

The MIC evaluation of the two molecules would provide interesting insights for speculating on the mechanisms behind the observed results. Urinary excretion of unmodified Empagliflozin corresponds to approximately 20% of the ingested dose (≈ 2 mg/day) [37]. Linagliptin is excreted in urine by reason of 7% of the daily dose (≈ 0.35 mg/day) [38]. This makes unlikely the possibility to reach the MIC for both drugs in the urinary tract, thus allowing us to exclude any direct effect of both compounds on bacterial growth and suggesting that the variations of the urinary communities derived from indirect effects, including the induced glycosuria and the modulation of the local inflammatory state.

Our hypothesis-generating study has some strengths. First, the inclusion of a control group at baseline allowed for a more robust comparison. Unlike other studies mainly focussing on the female gender, the recruitment of both male and female individuals in our clinical study prevents any gender-based bias and provide stronger evidence. In addition, the combined approach of high-resolution sequencing and Real-Time PCR ensured accurate characterisation of the microbial composition, and the PCA analysis allows a better global representation of microbiota components. However, there are also some limitations. The small sample size, while adequate to observe significant differences, does not allow finer stratified analyses based on age, sex, duration of diabetes, or presence of microvascular complications, thus limiting the generalisability of the results,

that should require further confirmation. The relatively short follow-up limits the possibility of evaluating the long-term effects of therapy on the urinary microbiota. In addition, we did not perform a functional analysis that could provide deeper insights into the metabolic functions associated with microbiota changes. Finally, the observational design of the study does not allow direct causation to be inferred; in particular, the association between Linagliptin use and microbiota restoration might be partly due to the improved glucose control or to potential changes in diet habits during the study. In the future, it will be important to conduct larger longitudinal studies that include clinical endpoints such as the frequency of urinary tract infections or other urogenital complications. It will also be interesting to evaluate whether similar effects are observed with other DPP-4i or in different therapeutic combinations, as well as to explore the effect on other microbial districts, such as the gut microbiota.

In conclusion, our data suggest that the Empagliflozin/Linagliptin combination preserves a urinary microbiota that is more similar to the eubiotic microbiota than Empagliflozin monotherapy. This effect could help improve the tolerability and safety of therapeutic strategies in subjects with T2D, opening new perspectives for an integrated approach to the management of microbial complications of the disease.

Author Contributions

Marco Calvigioni acquired data, designed this study, interpreted the results of the statistical analyses, produced tables and figures and critically reviewed the draughts of the manuscript. Edoardo Biancalana and Chiara Rossi performed the clinical studies. Diletta Mazzantini, Francesco Celandroni and Emilia Ghelardi made the statistical analyses and critically reviewed the manuscript. Anna Solini designed the study, got the funding and wrote the manuscript. Marco Calvigioni and Anna Solini are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Conflicts of Interest

Anna Solini has participated on speaker bureaus for Bayer, Lilly, Novo Nordisk, Sanofi. The other Authors have no conflicts to disclose.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Peer Review

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Information S1: dmrr70127-sup-0001-suppl-data.docx.

Figure S1: Flow diagram of the study.