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The role of urinary microbiota in primary and recurrent bladder cancer: insights from a propensity score matching study

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Abstract

Background Bladder cancer (BCa) is a common urinary malignancy with high recurrence rates in non-muscle invasive bladder cancer (NMIBC), posing significant clinical challenges. Emerging evidence links urinary microbiota to cancer progression; however, their role in BCa recurrence remains unclear. This study aimed to explore urinary microbiota differences between primary and recurrent BCa to identify potential microbiological markers and mechanisms associated with recurrence.

Methods Urine samples were collected from 170 BCa patients, including 125 with primary Bca(BCa_P) and 45 with recurrent BCa (BCa_R). All samples underwent 16 S rRNA gene sequencing, and clinical data were collected, including age, sex, body mass index (BMI), smoking history, pathological grade, and other biological characteristics. Propensity score matching (1:1 ratio, caliper = 0.02) minimized baseline differences, resulting in 39 matched pairs. Microbial diversity was analyzed using α and β diversity indices. Differential taxa were identified with Linear Discriminant Analysis Effect Size (LEfSe), and functional pathways were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt).

Results Alpha diversity was significantly higher in BCa_P than BCa_R, particularly in Chao1 indices. β diversity revealed distinct microbial structures (ADONIS, $P = 0.004$, $R^2 = 0.025$). At the phylum level, both BCa_P and BCa_R were dominated by Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria, with Firmicutes significantly higher and Bacteroidetes lower in BCa_R. At the genus level, BCa_P was enriched in Sphingomonas, Corynebacterium, Capnocytophaga, Massilia, and Aquabacterium, while BCa_R showed higher levels of Aeromonas, Cupriavidus, and Bradyrhizobium. Functional predictions revealed glucose metabolism and oxidative stress pathways enriched in BCa_R, while pollutant degradation and TCA cycle pathways were prominent in BCa_P.

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Conclusion These findings reveal significant differences in urinary microbiota compositions and functional profiles between primary and recurrent BCa patients, with recurrent cases exhibiting reduced microbial diversity and enrichment of potentially pathogenic communities, highlighting their potential roles in tumor progression and recurrence.

Trial registration Registered with the Chinese Clinical Trial Registry (ChiCTR2300070969) on April 27, 2023.

Keywords BCa, Urinary Microbiome, Recurrence, 16sRNA

Introduction

BCa is one of the most prevalent malignant tumors in the urinary system worldwide, characterized by high incidence and mortality rates [1]. Approximately 75% of patients are initially diagnosed with non-muscle invasive BCa (NMIBC) [2]. Between 50% and 70% of NMIBC patients experience recurrence within 5 years of treatment, and 10–30% may progress to muscle-invasive BCa (MIBC) [3], necessitating frequent cystoscopic monitoring and additional treatments. Despite advancements in treatment options, NMIBC recurrence continues to pose a significant clinical challenge. Thus, exploring novel non-invasive biomarkers and predictive tools for early detection and risk assessment of recurrence is particularly important.

In recent years, microbiome research has made substantial progress in the field of cancer [4]. The human microbiota is recognized for its crucial role in host health and disease conditions, particularly in immune regulation, metabolic control, and disease onset [5]. As a component of the urinary microenvironment, urinary microbiota may significantly influence the onset, progression, and recurrence of BCa [6]. However, existing studies primarily focus on the differences between BCa patients and healthy controls [7, 8], with few examining the variations in urinary microbiota between primary and recurrent BCa patients.

This study involved 170 BCa patients, including 125 patients with primary BCa and 45 with recurrent BCa. After propensity score matching analysis, 39 pairs of patients (1:1 matching) were successfully matched. The study aimed to compare the urinary microbiota characteristics between patients with primary and recurrent BCa, revealing the dynamic changes in urinary microbiota and their potential roles in cancer recurrence. By analyzing the diversity, community structure, and functional characteristics of the urinary microbiota, this study seeks to identify microbial biomarkers associated with BCa recurrence and explore their potential applications in clinical diagnosis and personalized treatment.

Patients and methods

Patient recruitment and sample collection

From May 2023 to March 2024, urine samples were collected from 170 BCa patients hospitalized in the Department of Urology at Changhai Hospital, Naval Medical University. Among them, 125 were patients with primary BCa, and 45 were patients with recurrent BCa during follow-up. All cases were histologically confirmed as urothelial carcinoma. To control for confounding factors and imbalance in baseline characteristics, a 1:1 propensity score matching analysis was performed. Following matching, 39 patients with primary BCa and 39 patients with recurrent BCa were included for further comparative analysis. All BCa cases were histologically confirmed as urothelial carcinoma. Subjects with a known history of sexually transmitted infections, recent urinary tract infections, or use of antibiotics or probiotics within one month were excluded. This study adhered to the ethical principles outlined in the Declaration of Helsinki. Patient recruitment and sample collection were approved by the Ethics Committee of Changhai Hospital, Naval Medical University (CHEC2023-062), with all participants signing informed consent forms before participation. This trial was registered on the Chinese Clinical Trial Registry (ChiCTR) with the registration number ChiCTR2300070969, registered on April 27, 2023. The registration details are publicly accessible on ChiCTR for verification and reference. Comprehensive clinical information, including age, sex, body mass index (BMI), tumor staging, and tumor grading, was recorded for all enrolled subjects. All participants provided clean midstream morning urine samples, which were immediately placed in sterile containers sealed to prevent environmental exposure, and rapidly frozen at -80 °C for further analysis. Prior to urine collection, participants were instructed to clean the periurethral area with antiseptic wipes (chlorhexidine) in a standardized manner: female participants wiped from front to back, while male participants retracted the foreskin to clean the glans. All sample processing steps were conducted in a biosafety cabinet to minimize airborne contamination. Laboratory equipment was regularly decontaminated using 10% bleach and UV sterilization before DNA extraction.

DNA extraction and sequencing

Total DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The integrity and concentration of the total DNA were assessed using a Thermo

NanoDrop 2000 UV spectrophotometer (Thermo Scientific, USA) and 1% agarose gel electrophoresis. The V3-V4 region of the 16 S rRNA gene was amplified using specific primers 341 F (5'-CCTACGGGRSGCAGCAG-3') and 806R (5'-GGACTACVVGGGTATCTAATC-3') with high-fidelity DNA polymerase from the KAPA HiFi Hotstart ReadyMix PCR kit. The cycling conditions for the first PCR were as follows: an initial denaturation at 98 °C for 3 min, followed by 25 cycles of 98 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min.For the second PCR, the amplicons from the first PCR were used as templates to attach sequencing adapters and sample indices. This was performed using the Nextera XT Index Kit (Illumina, USA). The second PCR was conducted under the following conditions: an initial denaturation at 98 °C for 3 min, followed by 12 cycles of 98 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min.

After the second PCR, the amplicons were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) to remove primer dimers and short fragments. The quality and size distribution of the amplicons were verified using 2% agarose gel electrophoresis to confirm the presence of the expected amplicon size. The library concentration was then quantified using a Qubit 2.0 fluorometer (Thermo Scientific, USA). Only libraries with a concentration ≥ 10 nM and no primer-dimer or non-specific bands were considered for sequencing. Paired-end sequencing was performed using the Illumina NovaSeq platform in PE250 mode.

16 S rRNA gene sequencing data analysis

Raw sequence data were analyzed using the Quantitative Insights Into Microbial Ecology (QIIME, V1.9.1) software package, with low-quality reads, primer contaminants, and barcode errors removed through quality control. Clean reads were further processed by filtering singletons (sequences with total abundance = 1) and clustered into Operational Taxonomic Units (OTUs) at a 97% similarity threshold using USEARCH (v10). Chimeric sequences were identified and removed using the UCHIME algorithm within USEARCH. Taxonomic assignments were performed using the Ribosomal Database Project (RDP) classifier (Version 18, August 14, 2020). All clean reads were mapped to the filtered OTU sequences to generate the final OTU table. Alpha diversity was evaluated using the rarefied OTU Tables (10,382 sequences per sample, determined via rarefaction curve analysis, see Fig. S1) and employed Chao1, Observed species, PD whole tree, and Shannon indices to measure species richness and diversity within samples. Beta diversity was calculated using weighted and unweighted UniFrac distances based on the rarefied OTU table and evaluated differences in microbial communities between samples and visualized through Principal Coordinate Analysis (PCoA). Microbial functions were also predicted using high-quality sequences via the PICRUSt method [9].

Statistical analyses

In this study, propensity score matching (PSM) was performed to control for potential confounding factors. The matching variables included age, sex, body mass index (BMI), tumor staging, tumor grading, and smoking status. A 1:1 nearest neighbor matching method was applied with a caliper width of 0.02. Following matching, statistical analyses were conducted to examine the microbiota characteristics and other variables. Analyses were performed using QIIME [10]and the R package (version 4.2.1). The Wilcoxon rank-sum test was used to identify differences in the microbiota, while an independent samples t-test, chi-square test, and Fisher's exact test were employed to analyze contingency tables. Spearman's rank test was conducted for correlation analysis. To identify taxa significantly associated with sample groupings, we performed Linear Discriminant Analysis Effect Size (LEfSe, V1.0) with a logarithmic LDA score threshold of >2.0. LEfSe combines non-parametric Kruskal-Wallis tests for feature selection and linear discriminant analysis (LDA) to estimate the effect size of differentially abundant taxa. For species-level differential analysis, we performed false discovery rate (FDR) correction and reported q-values instead of *p*-values. For beta-diversity comparisons, permutational multivariate analysis of variance (PERMANOVA, Adonis) and Multi-Response Permutation Procedure (MRPP) were applied to weighted and unweighted UniFrac distance matrices. The unweighted UniFrac metric evaluates differences in microbial diversity based solely on the presence or absence of taxa, while weighted UniFrac incorporates taxa abundances to assess overall compositional differences between groups. Both methods test the null hypothesis that microbial community structures do not differ between groups. PERMANOVA (Adonis) partitions variance across groups using a linear model (999 permutations), reporting the explanatory power (R^2) and significance (*p*-value). MRPP calculates the within- and between-group dissimilarities, providing a chance-corrected within-group agreement statistic (A-value) and significance. These analyses were implemented via the vegan package in R(v2.4.1). A *P*-value of less than 0.05 was considered statistically significant.

Results

Propensity score matching analysis of baseline characteristics and sequencing data analysis

In this study, 170 BCa patients were included, comprising 125 primary BCa patients (BCa_P) and 45 recurrent BCa patients (BCa_R). Before matching, significant differences

were observed between the BCa_P and BCa_R groups in terms of sex distribution ($p = 0.014$), pathological grade ($p < 0.001$), and tumor stage ($p = 0.009$). After propensity score matching, 39 pairs of patients were matched, and baseline characteristics were balanced between the groups ($p > 0.05$). Post-matching analysis revealed no significant differences in age, sex, BMI, smoking status, pathological grade, or tumor stage between the BCa_P and BCa_R groups, ensuring comparability (Table 1).

From the 78 cohort samples, we obtained a total of 2,700,958 high-quality sequences after quality control, yielding an average of 34,627 sequences per sample. Sequencing information for each sample is presented in Table S1. These sequences were clustered into 1,782 annotated operational taxonomic units (OTUs) based on a 97% similarity threshold. OTU data for each sample are presented in Table S2. The species accumulation curves for all samples reached an asymptote, indicating the adequacy of our sampling efforts (Fig. 1 A). The primary group contained 1,261 OTUs, whereas the recurrent group contained 1,122 OTUs, with 601 OTUs shared among all groups. These OTUs may reflect common mechanisms underlying the occurrence and development of BCa. The recurrent group contained 521 unique OTUs (Fig. 1B). The sequences from these specimens were classified into 2,059 phyla, 1,930 classes, 1,812 orders, 1,655 families, and 1,307 genera. The most abundant phyla detected were Firmicutes (43.66%), Proteobacteria (19.28%), Bacteroidetes (18.46%), and Actinobacteria (13.70%) (Fig. S2). The most abundant genus detected was *Prevotella* (12.05%), followed by *Streptococcus* (10.01%), *Corynebacterium* (7.49%), and *Escherichia/Shigella* (5.96%) (Fig. 1 C).

Urinary microbiota alpha and Beta diversity in primary and recurrent BCa patients

The analysis of the urinary microbiota alpha diversity in primary and recurrent BCa patients revealed significant differences in species richness and evenness. Specifically, the Chao1 index was significantly higher in the BCa_P group compared to the BCa_R group ($p = 0.031$, Fig. 2 A), indicating that the microbiota in primary BCa patients exhibited greater species richness. In contrast, the Good's coverage index was significantly higher in the BCa_R group ($p = 3.1e\text{-}06$, Fig. 2B), suggesting that the microbiota in the BCa_P group was more complex, with some rare species not fully covered in the analysis. While other diversity metrics (e.g., Simpson index, Observed Species index, PD Whole Tree index, and Shannon index) did not reach statistical significance (Fig.S3A-3D), the overall diversity indices were higher in the BCa_P group, suggesting a more complex and even microbial ecosystem in these patients.

Table 1 Baseline characteristics of patients with primary and recurrent BCa under the propensity score-matched cohort

Variables	Unmatched(n = 170)			Matched (n = 78)		
	BCa_P (n = 125)	BCa_R (n = 45)	*p*-value	BCa_P (n = 39)	BCa_R (n = 39)	*p*-value
Demographics/anthropometric						
Age yr (mean ± SD)	67.62 ± 11.77	67.13 ± 11.71	0.814	70.44 ± 9.41	67.95 ± 9.91	0.259
Male/female (No.)	107/18	31/14	0.014	28/11	31/8	0.429
BMI (kg/m2) (mean ± SD)	24.22 ± 3.44	23.95 ± 3.27	0.648	24.31 ± 3.77	24.22 ± 3.27	0.906
Pathological grade			< 0.001			1
Low grade	81 (64.8%)	42 (93.33%)		33 (7.69%)	3 (7.69%)	
High grade	44 (35.2%)	3 (6.67%)		36 (92.31%)	36 (92.31%)	
Biological characteristics			0.009			1
NMIBC	19 (15.2%)	15 (33.33%)		28 (71.79%)	28 (71.79%)	
MIBC	106 (84.8%)	30 (66.67%)		11 (28.21%)	11 (28.21%)	
Smoking status			0.203			0.645
Never smoker	64 (51.2%)	28 (62.22%)		24 (61.54%)	22 (56.41%)	
Ever smoker	61 (48.8%)	17 (37.78%)		15 (38.46%)	17 (43.59%)	

An independent samples t-test was used to compare age and BMI between the two groups. Other categorical data were analyzed using the chi-square. test and Fisher's exact test

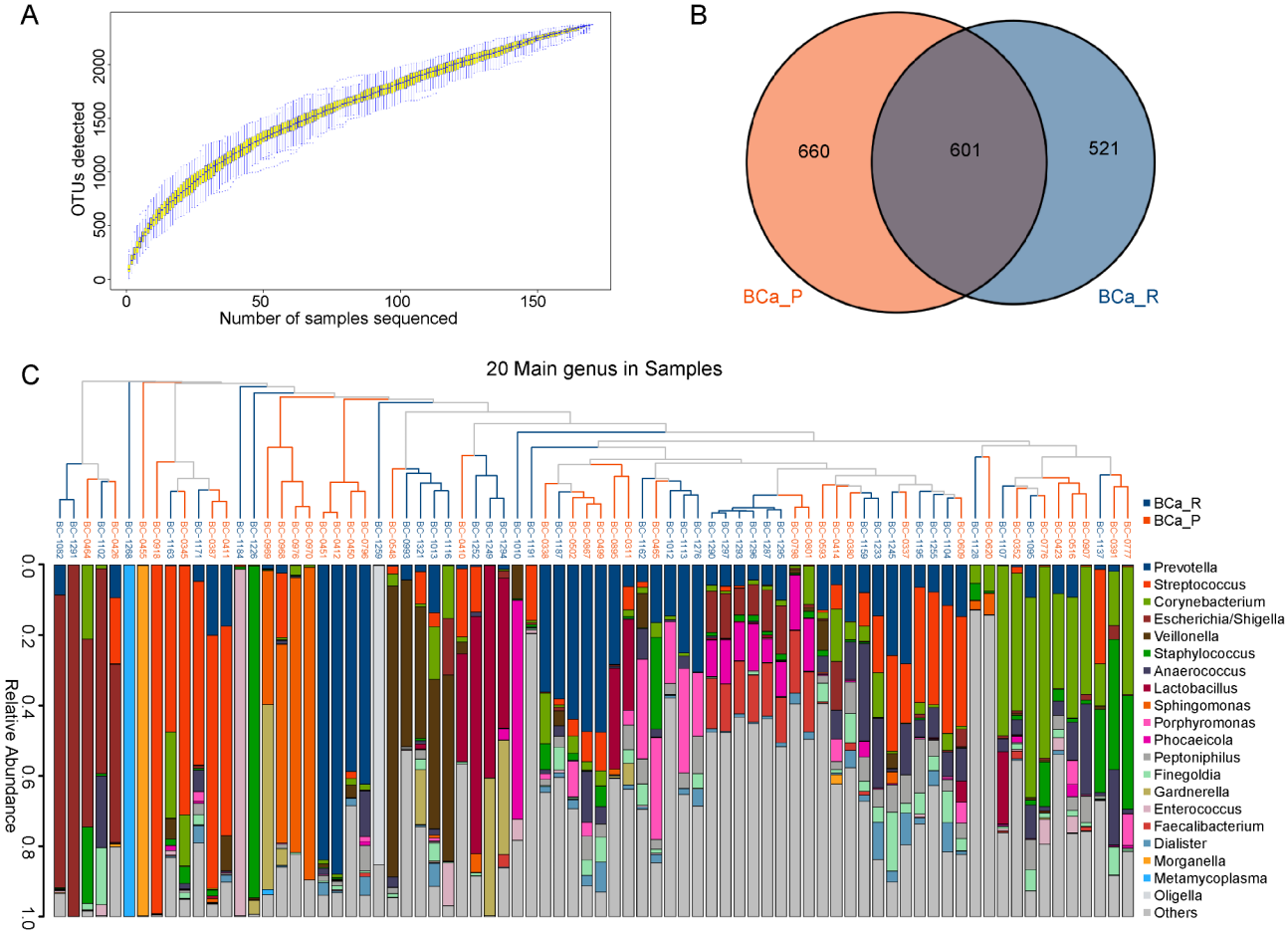

Fig. 1 Analysis of OTU Distribution and Microbial Composition in Primary and Recurrent BCa Patients. (**A**) Species accumulation curve showing the number of OTUs detected with increasing sample size. The curve initially rises sharply and then gradually levels off, indicating that sufficient sampling has been achieved. This suggests that further sampling will likely yield only a marginal increase in species detection. (**B**) Venn diagram showing the distribution of Operational Taxonomic Units (OTUs) in primary BCa patients (BCa_P, $n = 39$) and recurrent BCa patients (BCa_R, $n = 39$). A total of 660 OTUs were unique to the primary group, 521 OTUs were unique to the recurrent group, and 601 OTUs were shared between both groups, indicating differences in microbial community structure between primary and recurrent BCa patients. (**C**) Relative abundance of the 20 main genera in samples. The blue bars represent the Bca_R group, while the orange bars represent the Bca_P group. Each bar represents a sample, and the colors within the bars represent the relative abundance of different genera. The tree dendrogram on the left reflects hierarchical clustering of samples based on their microbiota composition

Using unweighted UniFrac distances (qualitative analysis), we observed a significant difference in β-diversity between primary and recurrent BCa patients (Adonis test, $P = 0.004$, $R^2 = 0.025$; Fig. 2C). In addition, ANOSIM analysis confirmed these results, showing significant differences between the two groups for unweighted UniFrac distances ($R = 0.055$, $P = 0.004$;Fig. S3E).The unweighted UniFrac heatmap (Fig. 2D) further corroborated this finding, demonstrating that samples within each group were more similar to one another than to samples from the other group. This indicates a distinct difference in the urinary microbiota community structure between the two groups. In contrast, the weighted UniFrac analysis did not reveal significant differences (Fig. S3F-3G). Additionally, results from the Multi-Response Permutation Procedure (MRPP) based on unweighted UniFrac distances also indicated significant differences in urinary microbiota communities between primary and recurrent BCa patients (A = 0.006596, $P = 0.005$; Table S3), consistent with the Adonis test. These findings suggest that there may be changes in the composition of the urinary microbiota associated with BCa_R, though further longitudinal studies are needed to confirm these associations and assess their potential role in disease progression.

Characterization and comparative analysis of urinary microbiota in BCa_P and BCa_R patients

Analysis of the urinary microbiota at the phylum and genus levels revealed significant differences between BCa_P and BCa_R(Fig. 3A and B). At the phylum level, the top four most abundant phyla in both groups were *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*. However, the proportion of *Firmicutes* was higher in BCa_R compared to BCa_P. At the genus level, species

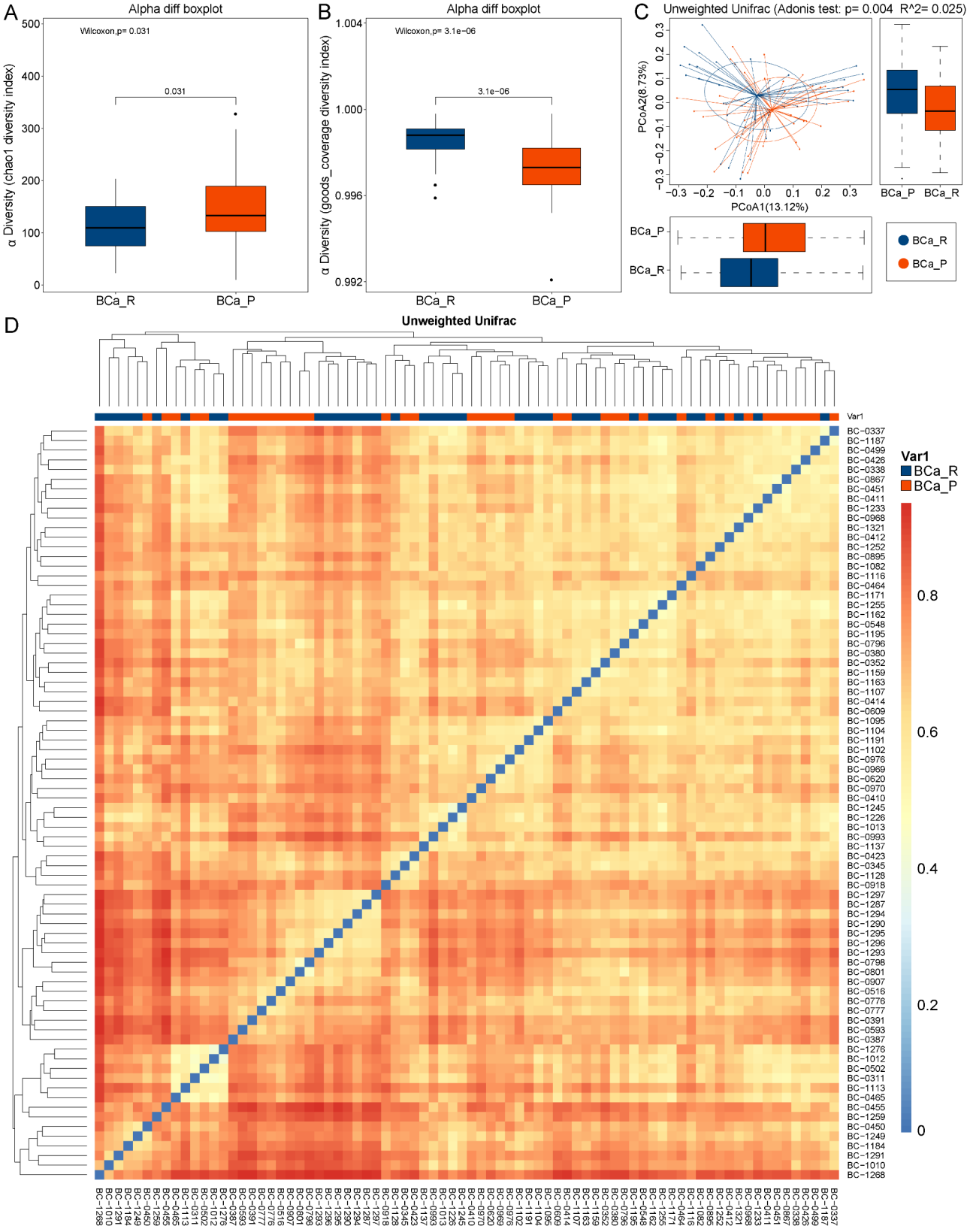

Fig. 2 (See legend on next page.)

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Fig. 2 Alpha and Beta Diversity Analysis of Urinary Microbiota in Primary and Recurrent BCa Patients. (**A**) Boxplot comparing the alpha diversity (Chao1 diversity index) between primary BCa patients (BCa_P, $n = 39$) and recurrent BCa patients (BCa_R, $n = 39$). Primary patients exhibited significantly higher alpha diversity than recurrent patients (Wilcoxon test, $p = 0.031$). (**B**) Boxplot comparing the alpha diversity (Good's coverage) between primary and recurrent BCa patients. A significant reduction in species diversity was observed in recurrent patients compared to primary patients (Wilcoxon test, $p = 3.1e\text{-}06$). (**C**) Principal Coordinate Analysis (PCoA) plot based on unweighted UniFrac distances, illustrating beta diversity differences between primary and recurrent BCa patients (ADONIS test, $p = 0.004$, $R^2 = 0.025$). The distinct clustering indicates significant differences in microbial community structure between the two groups. Boxplots on the right and below represent the distribution of UniFrac distances for both groups, further emphasizing the divergence in community composition. (**D**) Heatmap of the Unweighted UniFrac distance matrix, illustrating the microbial dissimilarity between primary and recurrent BCa patients. Red indicates higher similarity, and blue represents lower similarity

such as *Sphingomonas*, *Prevotella*, and *Corynebacterium* were enriched in the urine of BCa_P patients, while *Veillonella*, *Escherichia/Shigella*, and *Lactobacillus* were more abundant in recurrent BCa_R patients' urine.

LEfSe analysis (Fig. 3C) showed significant taxonomic and abundance differences in the urinary microbiota between BCa_P and BCa_R patients. The primary BCa group was enriched with genera such as *Corynebacterium*, *Capnocytophaga*, *Massilia* and Polynucleobacter. In contrast, the recurrent BCa group showed enrichment of genera like *Ralstonia*, *Aeromonas*, *Cupriavidus*, and *Bradyrhizobium*.

The phylogenetic tree in Fig. 3D further corroborates these findings, highlighting significant taxonomic divergence in microbial community structure between the two groups. Additionally, the relative abundance analysis of the top 20 differential genera (Fig. 3E; Table 4) provides further support for the LEfSe results, showing distinct microbial community profiles between the two groups. These findings indicate that the urinary microbiota undergoes significant changes during BCa recurrence, suggesting potential biomarkers and mechanisms that may contribute to tumor recurrence.

To further explore the interactions between urinary microbiota in BCa_P and BCa_R patients, we conducted Spearman correlation analysis on the top 30 differentially abundant genera (Fig. S4). This analysis revealed distinct patterns of microbial co-occurrence in the two patient groups. In the BCa_P group, many genera exhibited positive correlations, indicating a stable and cooperative microbial ecosystem. Genera such as *Aliarcobacter*, *Lactococcus*, *Lacticaseibacillus*, *Rouxiella*, and *Uruburuella* formed a strong positive correlation cluster. In the BCa_R group, positive correlations were also observed among genera like *Ralstonia and bradyrhizobium*, *Mesorhizobium* and *Atopostipes*. However, a significant number of negative correlations (red regions) were found between genera such as *Ralstonia* and *Cupriavidus*.

Predictive function analysis

We used PICRUSt to predict the functional composition in the urine microbiota of BCa patients based on 16 S rRNA sequencing data, observing significant differences in multiple KEGG functional pathways between primary and recurrent BCa patients (Fig. 4). The microbiota in the BCa_P group was significantly enriched in functional pathways associated with metabolic adaptation, including *carbon fixation pathways*, *the tricarboxylic acid (TCA) cycle*, and *prokaryotic cell cycle regulation pathways*. In contrast, the microbiota in the BCa_R group showed significant enrichment in functional pathways related to glucose metabolism and oxidative stress, such as *amino sugar* and *nucleotide sugar metabolism*, *the pentose phosphate pathway*, and *starch and sucrose metabolism*. These findings suggest that microbial communities in BCa_P and BCa_R patients display distinct functional profiles, reflecting the varying metabolic needs and ecological adaptations of the microbiota.

Discussion

BCa is a heterogeneous disease characterized by distinct clinical and molecular features that may evolve over time, particularly during recurrence. Understanding the role of urinary microbiota in both primary and recurrent BCa is crucial for identifying potential microbial signatures that could serve as biomarkers or therapeutic targets. In this study, we investigated the urinary microbiota in BCa_P and BCa_R patients, employing propensity score matching to balance baseline characteristics and high-throughput sequencing to analyze microbial diversity. Our results demonstrate significant differences in urinary microbiota composition between BCa_P and BCa_R patients, highlighting potential microbial-driven mechanisms underlying tumor progression and recurrence.

Our analysis revealed significant differences in alpha diversity between BCa_P and BCa_R patients. The Chao1 index, which measures species richness, was significantly higher in the BCa_P group, suggesting that BCa_P is associated with a more diverse microbial community. In contrast, the Good's coverage index was significantly higher in the BCa_R group, indicating that the urinary microbiota in recurrent BCa is less diverse or dominated by specific microbial taxa. This dysbiosis, characterized by reduced microbial diversity, may contribute to the recurrence of BCa. Beta diversity analysis further supported these findings, revealing significant structural differences in the urinary microbiota between BCa_P and BCa_R patients. The unweighted UniFrac analysis, which accounts for phylogenetic relationships among microbial communities, demonstrated distinct microbial profiles

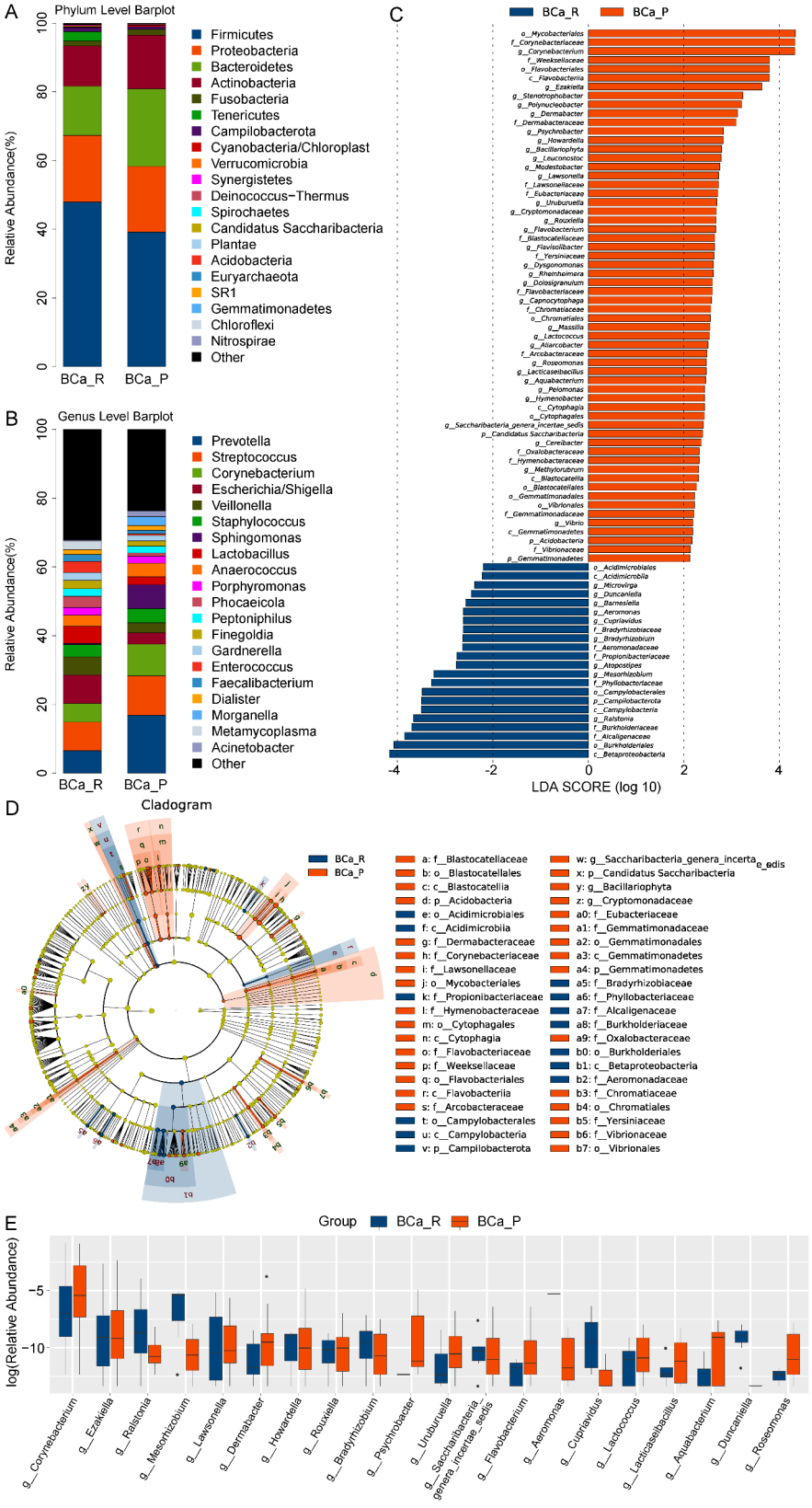

Fig. 3 (See legend on next page.)

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Fig. 3 Taxonomic Composition and Differential Analysis of Urinary Microbiota in BCa Patients. (**A**) Phylum-level bar plot showing the relative abundance of microbial communities in urine samples from primary BCa (BCa_P) and recurrent BCa (BCa_R) patients. Differences in the distribution of major phyla such as *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and others are evident between the two patient groups. (**B**)Genus-level bar plot representing the relative abundance of microbial genera in BCa_P and BCa_R patients. Dominant genera such as *Prevotella*, *Streptococcus*, *Corynebacterium*, and *Escherichia/Shigella* show distinct abundance patterns between the two groups. The "Other" category represents less abundant genera. (**C**) Differential abundance analysis of microbial genera using Linear Discriminant Analysis (LDA) Effect Size (LEfSe) to identify significant bacterial taxa between primary BCa (BCa_P) and recurrent BCa (BCa_R) patients. The plot shows LDA scores (log10) for the top genera, highlighting those with the most significant differences in abundance between the two groups. Positive LDA scores represent genera more abundant in BCa_P, while negative scores indicate genera more abundant in BCa_R. Statistically significant genera are shown with distinct color coding: blue for BCa_R and orange for BCa_P. (**D**) Cladogram representing the microbial taxonomic differences between primary BCa (BCa_P) and recurrent BCa (BCa_R) patients. The taxonomic tree illustrates the hierarchical distribution of bacterial taxa, with the significant differences in relative abundance highlighted using color coding: blue for BCa_R and orange for BCa_P. The branches represent various taxonomic levels, from phylum to genus, with the most significant taxa differences indicated by the larger branches and outermost nodes. (**E**) Boxplot showing the log-transformed relative abundance of selected microbial genera in urine samples from primary BCa (BCa_P) and recurrent BCa (BCa_R) patients. Significant differences in the relative abundance of genera such as *Corynebacterium*, *Ezakella*, *Roseomonas*, and others are observed between the two groups, with statistical differences indicated by the boxplot distributions. Blue represents BCa_R and orange represents BCa_P

in the two groups. This observation suggests that BCa recurrence is associated with shifts in microbial community structure, potentially driven by changes in the tumor microenvironment that alter microbial colonization and dynamics.

Previous studies have demonstrated that the loss of gut microbiota diversity is associated with diseases such as irritable bowel syndrome, inflammatory bowel disease, and colorectal cancer, where dysbiosis disrupts the mucosal barrier, leading to inflammation and potential tumorigenesis [12–14]. However, diversity changes in urinary microbiota show a more complex pattern in urogenital diseases. For example, urgency urinary incontinence is associated with increased microbial diversity [14], whereas reduced diversity has been observed in overactive bladder syndrome [15]. These discrepancies may reflect the distinct responses of urinary microbiota to various pathological conditions. Regarding BCa, some studies have reported increased urinary microbiota diversity in BCa patients compared to healthy controls [16], while others found no significant differences [17]. Qiu et al. observed that lower urinary microbiota diversity was associated with prolonged recurrence-free survival [18], contrasting with our findings. In our study, the reduced alpha diversity observed in the recurrent group may correlate with disease recurrence, as lower diversity could result in diminished metabolic and immune support, creating an environment conducive to cancer progression [19]. Although current evidence remains inconclusive, we hypothesize that, akin to gut microbiota dysbiosis, urinary microbiota imbalances may represent a potential factor in BCa development. Future studies are needed to further investigate the quantitative and functional roles of urinary microbiota in BCa.

At the phylum level, both BCa_P and BCa_R patients' urinary microbiota were dominated by *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria*, consistent with the typical composition of the human urinary microbiota [20]. However, the relative abundance of Firmicutes was significantly higher and Bacteroidetes significantly lower in BCa_R patients, a dysbiosis pattern associated with poor prognosis in other cancers [21].

At the genus level, distinct microbial enrichments were observed between the two groups. In BCa_P patients, the high abundance of *Sphingomonas* may reflect exposure to polycyclic aromatic hydrocarbons (PAHs) derived from smoking, environmental pollutants, and dietary intake [22]. Studies have linked environmental exposure to chemicals or heavy metals with increased BCa incidence [23], and *Sphingomonas* is known to metabolize these substances [24, 25]. This may explain why most studies have identified *Sphingomonas* as a core bacterium in bladder cancer patients [22, 26]. Additionally, *Corynebacterium* was significantly enriched. As a common component of the male urinary microbiota [27], *Corynebacterium* has been linked to chronic urethral inflammation [28] and biofilm formation [29], both of which are implicated in cancer initiation [30, 31]. Furthermore, the ability of *Corynebacterium* to metabolize urea and other nitrogen-containing compounds may alter the bladder's metabolic environment, potentially promoting early tumorigenesis [32]. Interestingly, recent study observed an increase in *Corynebacterium* following the wash-out period between the sixth BCG instillation and the first maintenance cycle, suggesting that its abundance rises as the bladder microbiota shifts to a more commensal state post-treatment [33]. This highlights the complex role of *Corynebacterium* in bladder cancer progression and recovery, suggesting that while it may contribute to early tumorigenesis, its involvement in cancer recurrence is likely modulated by treatment-induced alterations in the urinary microbiota.

Notably, *Capnocytophaga*, an opportunistic pathogen that has been demonstrated to promote tumor invasion and metastasis in oral cancer [34], was significantly enriched in BCa_P patients. In addition, *Massilia* and *Aquabacterium* also exhibited high abundance in BCa_P patients. These genera are well-known for their metabolic versatility, including the degradation of polycyclic aromatic hydrocarbons (PAHs) and the removal

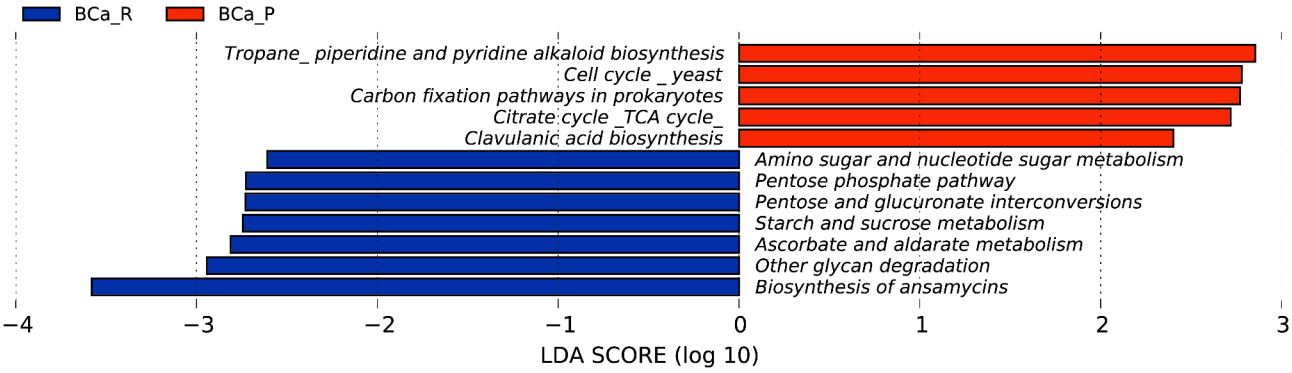

Fig. 4 Functional Pathway Analysis of Urinary Microbiota in Primary and Recurrent BCa Patients. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) plot showing the differential abundance of microbial pathways between primary BCa (BCa_P) and recurrent BCa (BCa_R) patients. The LDA scores (log10) represent the significance of each metabolic pathway, with positive scores indicating pathways more enriched in BCa_P and negative scores indicating pathways more abundant in BCa_R. The pathways are color-coded with blue for BCa_R and orange for BCa_P

of environmental pollutants [35, 36]. Collectively, these findings suggest that these genera may influence BCa development through metabolic reprogramming and modulation of the tumor microenvironment.

In BCa_R patients, genera such as *Aeromonas*, *Cupriavidus*, and *Bradyrhizobium* were significantly enriched. *Aeromonas* can induce persistent tissue damage and inflammation, leading to epithelial regeneration, genomic instability, and increased mutational risk [37]. *Cupriavidus*, known for its ability to degrade pesticide residues such as hexachlorocyclohexane [38], may modulate the metabolic environment to support cancer recurrence. *Bradyrhizobium* has been associated with a variety of malignancies, indicating a substantial correlation. Specifically, *Bradyrhizobium japonicum* has been reported to be enriched in the gut microbiome of individuals with lung cancer, potentially facilitating tumorigenesis through the induction of genomic instability [39]. Additionally, the presence of *Bradyrhizobium* has been confirmed in solid tumor types, including cervical, ovarian, and endometrial cancers [40], where it exhibits an inverse relationship with the androgen receptor, thereby possibly modulating the clinical trajectory of prostate cancer via the regulation of hormone signaling pathways [41]. In the context of pancreatic ductal adenocarcinoma (PDAC), *Bradyrhizobium* has been noted to be particularly abundant in the pancreatic head region [42], implying a role in the modulation of local microbiota that could influence tumor recurrence and progression. The significant enrichment of these microbial genera and their associated mechanisms underscores the intricate interplay between the microbiome and the tumor microenvironment, providing critical insights into the pathophysiological underpinnings of tumor recurrence and paving the way for the development of microbiome-informed therapeutic interventions.

Recent studies have shown that probiotics, particularly *Lactobacillus* species, can exhibit anticancer properties by competing with pathogenic strains involved in carcinogenesis or by producing regulatory substances [43, 44]. In our study, the positive correlation between *Lactococcus* and *Lacticaseibacillus* in the urine microbiota of treatment-naïve bladder cancer (BCa_P) patients suggests a synergistic interaction that may stabilize the urinary microenvironment and modulate tumor progression. The co-occurrence of these genera likely represents a cooperative network with combined immunomodulatory and antiproliferative functions. Research has shown that lactic acid-producing bacteria, including *Bifidobacterium*, *Lactobacillus*, and *Lactococcus*, provide significant health benefits and are associated with bladder cancer [45]. Specifically, has been shown to inhibit cancer cell proliferation and the production of pro-inflammatory cytokines [46]. Additionally, has been found to induce apoptosis in colon cancer cells and trigger the release of DAMPs, indicative of immunogenic cell death [47]. This synergy aligns with findings suggesting that probiotic combinations can enhance antitumor effects by activating apoptotic pathways and inhibiting inflammasome signaling [48]. These observations highlight the potential cooperative roles of *Lactococcus* and *Lacticaseibacillus* in early bladder cancer, though their functional contributions need further validation in the urinary niche.

Using PICRUSt-based functional predictions, we identified significant differences in KEGG pathways between BCa_P and BCa_R patients. Metabolic reprogramming, a hallmark of cancer progression, manifests as altered nutrient utilization and biosynthesis, which may also drive immune evasion or impair immune surveillance [49, 50]. In BCa_P patients, microbial communities were enriched in pathways related to metabolic adaptation and early tumorigenesis, including *carbon fixation pathways* and *the TCA cycle*. These microbial pathways may influence the host's metabolic environment, contributing to tumor initiation by modulating energy metabolism and immune responses [51]. In contrast, BCa_R patients

showed enrichment in pathways associated with glucose metabolism and oxidative stress, such as *amino sugar* and *nucleotide sugar metabolism*, *the pentose phosphate pathway*, and *starch and sucrose metabolism*. These functional shifts likely reflect microbial adaptation to the metabolic demands in recurrent cancer, potentially supporting sustained tumor growth [52]. These functional differences reflect the dynamic metabolic and environmental adaptations of microbial communities in primary versus recurrent BCa, providing valuable insights into the potential role of microbiota in tumor development and recurrence.

This study provides compelling evidence of dynamic changes in urinary microbiota during BCa recurrence. The observed microbial differences between BCa_P and BCa_R patients may serve as non-invasive biomarkers for predicting recurrence. Future studies are needed to validate these findings and explore the functional consequences of these microbial shifts in BCa progression. Additionally, microbiota-targeted interventions, such as probiotics or microbiota modulation [53], may offer novel therapeutic strategies for preventing recurrence and improving patient outcomes.

Despite its strengths, this study has several limitations. First, the sample size was relatively small, which, although adjusted using propensity score matching, may limit the generalizability of our findings. Second, while 16 S rRNA sequencing provided valuable insights into microbial composition and function, it does not capture the full genomic or transcriptional activity of these microbes. Integrative approaches, including metagenomics and transcriptomics, are needed to comprehensively characterize the functional roles of microbiota in BCa. In addition, as an observational study, this work cannot establish causality between microbiota changes and BCa progression. Future large-scale, multicenter prospective studies and experimental validation are warranted to elucidate the specific mechanisms linking urinary microbiota to BCa development and recurrence.

Furthermore, the choice of urine collection method should also be considered. Our study utilized midstream morning urine samples, which are clinically non-invasive and widely adopted. However, this approach carries a potential risk of contamination from skin or distal urethral microbiota, possibly leading to overrepresentation of commensal bacteria (e.g., Lactobacillus, Corynebacterium) in the analyzed community [22, 54]. Although we followed standard cleansing protocols to minimize contamination, catheterized urine samples would more accurately reflect the bladder microbiota [55]. Future studies may consider comparing both sampling methods to quantify the impact of contamination in similar settings. Additionally, the absence of negative controls in this study is a limitation. Given the low bacterial load in urine samples, the risk of contamination from environmental sources or during laboratory processing cannot be fully excluded. Future research should incorporate negative controls to better differentiate between true microbial signatures and potential contaminants.

Conclusion

In summary, this study systematically compared the urinary microbiota of primary and recurrent BCa patients, unveiling significant changes in the microbiome during recurrence. We found that dynamic changes in specific microbial communities are closely related to cancer recurrence, providing new insights into understanding the potential biological mechanisms of BCa recurrence. Future research should focus on exploring the functional roles of these microbial communities in BCa recurrence and how the regulation of these key genera could facilitate early diagnosis and personalized treatment of BCa recurrence.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions

ZZS, XCL, and ZSX designed the project; SZY and WMY managed the project; XJS and XX collected samples and performed the clinical study; SZY and XJS managed all the experiments including DNA extraction experiments, library construction, and sequencing; XX and ZJP designed the analysis; SZY and WMY analyzed the data and wrote the manuscript; ZZS, XCL, and ZSX revised the manuscript. All authors read and approved the final manuscript.

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Data availability

The dataset(s) supporting the conclusions of this article are available in the NCBI repository, PRJNA1155298 (http://www.ncbi.nlm.nih.gov/bioproject/PRJNA1155298).All other data relevant to the study were included in the article or uploaded as supplementary information files.

Declarations

Ethics approval and consent to participate

Informed written consent was obtained from all patients at the time of enrollment. The study was conducted in accordance with the ethical principles outlined in the Declaration of Helsinki for research involving human subjects and received approval from the Ethics Committee of Changhai Hospital, Naval Medical University (Approval No. CHEC2023-062).

Consent for publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare no competing interests.

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