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Review Article

Possibilities and limitations of using low biomass samples for urologic disease and microbiome research

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ABSTRACT

With the dogma of sterile urine no longer held as truth, numerous studies have implicated distinct changes in microbial diversity and composition to diseased subgroups in both benign and malignant urological diseases, ranging from overactive bladder to bladder and prostate cancer. Further facilitated by novel and effective techniques of urine culture and sequencing, analysis of the genitourinary microbiome holds high potential to identify biomarkers for disease and prognosis. However, the low biomass of samples included in microbiome studies of the urinary tract challenge researchers to draw definitive conclusions, confounded by technical and procedural considerations that must be addressed. Lack of samples and adequate true negative controls can lead to overestimation of microbial influence with clinical relevance. As such, results from currently available studies and assessment of their limitations required a thorough understanding. The purpose of this narrative review was to summarize notable microbiome studies in the field of urology with a focus on significant findings and limitations of study design. Methodological considerations in future research are also discussed.

1. Introduction

Disease of the genitourinary tract has been traditionally associated with chronic inflammation, with epidemiological studies connecting underlying prostatitis to changes in the tumor microenvironment and subsequent pathogenesis.[1,2] With urine no longer established as a sterile environment as previously believed, distinct bacterial flora in the bladder and prostate of males with disorders of the urinary tract have been identified, with microbial dysbiosis putatively implicated as a cause of inflammation and clinical manifestation.[3] Fueled by advances in assay techniques including 16S ribosomal RNA (rRNA) and shotgun metagenomic sequencing, microbial taxonomies stratified by abundance and diversity can be used as potential biomarkers for identifying the presence and prognosis of disease as well as response to treatment.[4,5]

Although it is widely appreciated that both direct and indirect microbiomes can influence host immunity and harbor proneoplastic and anti-tumorigenic capabilities, identified uropathogens differ greatly between studies with variable associative significance.[6,7] These disparities in microbiota taxonomies are partly due to different sample types and assay techniques, not to mention probable sampling bias and contamination factors that might have been overlooked. As such, the significance and limitations of current microbiomes studies must be carefully addressed.[7,8] Here, we reviewed notable literature in different fields of urology, encompassing benign prostatic hyperplasia (BPH), lower urinary symptoms (LUTS), chronic prostatitis (CP) or chronic pelvic pain syndrome (CPPS), urolithiasis, as well as bladder and prostate cancer. Limitations of current studies that should be considered in future research were also examined.

2. Microbiomes of urologic disease

2.1. BPH/LUTS and CP/CPPS

BPH is a very common cause of LUTS in elderly male patients.[9] It is associated with systemic inflammation and oxidative stress

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promoted by metabolic syndrome linked to the development of BPH and LUTS.[10] Since prostatic inflammation has been suggested to play a major role in the progression of BPH, the urinary microbiome has been suspected to participate in the pathogenesis of BPH[3,11] (Table 1). Several studies have evaluated the association between the microbiota in BPH and male LUTS,[12–16] with samples obtained from both midstream[13,14,16] and catheterized urine.[13] While certain bacterial species including *Eubacterium* and *Defluviicoccus* have been implicated in BPH, most studies show bacterial species ubiquitously identified regardless of BPH with no α-diversity.[17] In addition, midstream "clean catch" urine failed to show adequate association with LUTS and increasing IPSS scores.[13] Most studies preferred such collection methods as opposed to catheterized urine due to ease of collection. However, *Lachnospiraceae* has shown a promising protective effect against BPH. It shows decreased abundance in BPH subjects than in cancer patients[12] and healthy controls.[14] Jain et al have also found that *Escherichia coli* isolated from BPH tissues can induce inflammation and DNA damage in *in vitro* prostate epithelial cells, corroborating the connection between local microbiome-mediated inflammation and BPH/LUTS progression.[15]

National Institutes of Health (NIH) classification has defined CP/CPPS disorder as urologic pain or discomfort in the pelvic region with urinary symptoms and/or sexual dysfunction for at least three of the previous six months excluding other sources of pelvic pain, including urinary tract infection, anatomic abnormalities, cancer, and neurological disorders.[18] Although there are overlapping symptoms between CP/CPPS and bacterial prostatitis or sexually transmitted infections, the presence and type of bacteria on conventional culture did not correlate with the presence or severity of CP/CPPS.[19] Several studies have evaluated the diversity of microbiome in patients with CP/CPPS patients in comparison with that of controls[2,11,17,20–23] and found that bacterial species are increased in the urine or seminal fluids of patients with CP/CPPS, although definitive patterns are lacking. Elevations of multiple bacterial taxa including *Clostridia* and *Bacteroidia*[20] in urine and *Achromobacter, Stenotrophomonas,* and *Brevibacillus*[22] in seminal fluids have been identified in patients of CP/CPPS, whereas levels of *Lactobacilli* are decreased in patients with CP/CPPS.[20,21]

2.2. OAB/UUI and IC/BPS

The etiology of an overactive bladder (OAB) has not been completely elucidated yet. According to International Urogynecological Association (IUGA)/International Continence Society (ICS), OAB is defined based on clinical symptoms such as urinary urgency usually associated with high urinary frequency and nocturia with or without urgency urinary incontinence (UUI) in the absence of UTI or other identified pathologies.[24] Therefore, a negative result in urine culture is essential for diagnosing OAB. As the dogma of sterile urine has been debunked, several attempts have been made to evaluate the urinary microbiome from OAB patients utilizing gene sequencing[1,17,25–30] (Table 2). Multiple studies assessing microbiomes in catheterized urine have shown elevated levels of *Actinobaculum* and *Aerococcus* in women with OAB and UUI.[25,26,29] Such elevation might be associated with the severity of symptoms.[30]

IC/BPS (interstitial cystitis/bladder pain syndrome) is a chronic painful bladder condition without other causes such as acute or recurrent infection, cancer, radiation-induced injury, or medication-induced injury. Although the diagnostic criteria of the European Society for the Study of IC/BPS (ESSIC) and the American Urological Association (AUA) are the most widely adopted criteria for IC/BPS, there is no single consensus definition for IC/BPS. The ESSIC has defined IC/BPS as chronic (>6 months) pelvic pain, pressure, or discomfort perceived to be related to the urinary bladder, accompanied by at least one or the other urinary symptoms such as a persistent urge to void or high frequency without other probable causes.[31] Several studies have shown differences in the diversity of microbiome between IC/BPS patients and asymptomatic controls[17,32–36] with variable results, including an overall increase of *Lactobacillus* genus in IC groups[34] but decreased levels of *Lactobacillus acidophilus*[33] without showing overall differences in fungal composition[35] or presence of Hunner lesions.[36]

2.3. Urolithiasis

The relationship between microbiota and urinary stone formation is relatively well-known (Table 3). For example, struvite stones are strongly associated with urea-splitting microbiota including *Proteus mirabilis* which can induce alkaline urinary environments, resulting in the crystallization of calcium, magnesium, and phosphate in the urine.[37] Several studies have reported the connection of urolithiasis with *Oxalobacter formigenes*, a Gram-negative, obligate anaerobe in intestinal microbiota.[17] By reducing oxalate absorption but stimulating oxalate secretion by the intestinal mucosa, *Oxalobacter* can reduce urinary oxalate excretion. Such oxalate-degrading ability of *Oxalobacter* suggests that intestinal depletion of *Oxalobacter* is associated with the generation of calcium oxalate urinary stone and that *Oxalobacter* could function as a probiotic with a potential role in treating hyperoxaluria.

In addition, diet is known to be a meaningful risk factor for stone formation, leading to an increased attention to indirect gut microbiota. With the development of next-generation sequencing (NGS) technology, several studies have analyzed intestinal microbiome from kidney stone patients and healthy controls.[17,38–42] Microbiota associated with short chain fatty acid (SCFA) production are decreased in patients with renal stones, putatively hindering the protective role of SCFA in maintaining gut barriers and decreasing systemic inflammation.[40,41]

2.4. Bladder cancer

Emerging evidence supports the pro-carcinogenic role of local microbial populations in the genitourinary tract. However, urine samples containing microorganisms (or microbial fragments and/or DNA) are limited in determining the "localization of bacteria in situ" to specific anatomical sites in the urethra, bladder, ureter, and/or kidney.[6] For this reason, research studies on the microbiome in urothelial cell carcinoma have been mostly limited to bladder cancer (BCa). While notable risk factors such as smoking, chemical exposure, and radiation therapy have been identified, it has been hypothesized that chronic infection might drive the development of BCa, as exemplified in *Schistosoma* mediating the production of N-nitrosamine, a well-described carcinogen.[43] Urinary microbiome of the bladder might play multiple roles in BCa pathogenesis and progression notably by building a biofilm barrier in the urinary tract epithelium, maintaining symbiosis with potential pathogenic bacteria, and disintegrating harmful metabolites[44] (Table 4).

Parra-Grande et al have reported that the amount of *Actinobacteria* is much higher in non-neoplastic bladder mucosa specimens than in tumor tissues,[45] supporting the hypothesis that *Actinomycete*-rich microbes associated with a lower incidence of BCa in women might have a preventive effect against BCa. Pederzoli et al[46] have demonstrated noticeable cluster differences in urine and tissue samples between males and females, implicating intersexual differences in microbiome that may explain the reduced prevalence of BCa in females due to the overall bladder microenvironment.

Table 1
Summary of studies on BPH/LUTS and CP/CPPS

Study	Sample size	Material	Analysis technique	Significant findings	Limitations
BPH/LUTS					
Yu et al (2015)[12]	21 BPH compared to 13 PCa subjects	Urine, seminal fluid, expressed prostatic secretion (EPS)	16S rRNA gene sequencing with PCR-DGGE analysis	BPH more likely to harbor increased *Eubacterium, Defluviicoccus* and less Bacteroidetes bacteria, *Alphaproteobacteria, Firmicutes bacteria, Lachnospiraceae, Propionicimonas, Sphingomonas, Ochrobactrum*	Limited samples
Bajic et al (2020)[13]	28 BPH compared to 21 controls	Midstream voided urine, Catheterized urine	16S rRNA gene sequencing and EQUC	Symptom severity based on IPSS scores significantly associated with detectable bacteria on catheterized urine.	Midstream urine inadequate to sample microbiome.
Holland et al (2020)[14]	30 men with LUTS	DNA from urine and fecal samples	16S rRNA gene sequencing	*Lachnospiraceae Blautia* showed protective effect against LUTS, especially bother components in IPSS, with correlation sustained at different levels of IPSS severity.	Limited sample, patients initially selected for biopsy, midstream urine
Jain et al (2020)[15]	36 BPH	DNA and sections from resected tissue	Culture and/or V3 16S rRNA gene sequencing	Inflammation identified in all BPH tissue, with *Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes* most commonly identified in V3 16S rRNA gene sequencing. *E.coli* isolated from BPH induced NF-κB activation and DNA damage in vitro.	Limited samples, multiple bacteria present with variable levels at different regions of same sample
Lee et al (2021)[16]	77 BPH and 30 controls	Midstream voided urine DNA	16S rRNA amplicon sequencing.	*Lactobacillus, Staphylococcus, Bacillus, Faecalibacterium, Listeria, Enhydrobacter, Pseudomonas, Neisseria, Phascolarctobacterium, Dolosigranulum, Haemophilus, [Ruminococcus] torques, Bamesiella, Finegoldia, Prevotellaceae NK3B31* group found in relative abundance in BPH	Cross-sectional study, no α-diversity, voided urine only
CP/CPPS					
Shoskes et al (2016)[20]	25 CP/CPPS and 25 controls	Urine DNA	16S rRNA gene sequencing	17 taxa over-represented in CP/CPPS including *Clostridia* and *Bacteroidia* and 5 under-represented including *Bacilli*, with increased overall bacterial diversity vs. control.	Cross-sectional design
Mändar et al (2017)[21]	21 CP/CPPS and 46 controls	Seminal fluid DNA	16S rRNA gene sequencing	CP/CPPS group: - more *Proteobacteria* - less *Lactobacilli* (especially *Lactobacillus iners*)	Lifestyle confounding factors not considered
Choi et al (2020)[22]	17 CP/CPPS and 4 controls	Seminal fluid	Bacterial culture and DNA pyrosequencing	CP/CPPS group: - more *Achromobacter, Stenotrophomonas, Brevibacillus*	Small sample, contaminants not controlled
Suarez et al (2021)[2]	5 men with CP/CPPS and 5 controls	Urine seminal fluid	Sequencing and Nitric oxide levels and proinflammatory cytokines in seminal and serum	The microbiota present in the semen and urine Samples of fertile men presents more operational taxonomical units. Less microbial diversity could be associated with CP symptoms.	Small sample
Kogan et al (2021)[23]	170 with CP/CPPS	Post-massage urine (VB3)	Meares–Stamey test	In patients with CP/CPPS, a predominance of anaerobes or a combination of aerobes and anaerobes in a titer of $>10^3$ colony-forming units per mL in post-massage urine is associated with worse clinical status.	Cluster analysis not done

Table 2
Summary of studies on OAB/UUI and IC/BPS

Study	Sample size	Material	Analysis technique	Significant findings
OAB/UUI				
Hilt et al (2014)[25]	41 women with OAB and 24 controls	Catheterized urine	Standard urine culture 16S rRNA gene sequencing and EQUC	Both OAB and control group - *Lactobacillus, Corynebacterium, Streptococcus, Actinomyces, Staphylococcus, Bifidobacterium* spp. only OAB group: - *Aerococcus, Actinobaculum*
Pearce et al (2014)[26]	23 women with UUI and 25 controls	Catheterized urine	16S rRNA gene sequencing and EQUC	UUI group: - more *Actinobaculum, Actinomyces, Aerococcus, Arthrobacter, Corynebacterium, Gardnerella, Oligella, Staphylococcus, Streptococcus* - less: *Lactobacillus*
Karstens et al (2016)[27]	10 women with daily UUI and 10 controls	Catheterized urine	16S rRNA gene sequencing	UUI group: - more *Sphingomonadales, Chitinophaga, Brevundimonas, Cadidatus Planktoluna, Alteromonadaceae, Elizabethkingia, Methylobacterium, Caldicellulosiruptor, Stenotrophomonas.* - less *Prevotella, Comamonadaceae, Nocardioides, Mycobacterium*
Thomas-White et al (2017)[28]	74 women with UUI and 60 controls	Catheterized urine	16S rRNA gene sequencing	Hormone-negative women: - less Lactobacillus, Gardnerella
Wu et al (2017)[29]	30 women with OAB and 25 controls	Catheterized urine	16S rRNA gene sequencing	OAB group: - more *Sneathia, Staphylococcus, Proteus, Helcococcus, Gemella, Mycoplasma, Aerococcus* - less *Prevotella, Dialister, Fusobacterium, Jonquetella, Campylobacter, Finegoldia, Anaerococcus, Lactobacillus, Pyramidobacter, Ureaplasma, Enterococcus, Novosphingobium, Lactococcus*
Fok et al (2018)[30]	126 Women undergoing POP/SUI surgery	Catheterized urine Vaginal and perineal swab	16S rRNA gene sequencing	Higher OABq symptom severity score: - more *Atopobium vaginae, Finegoldia magna*
IC/BPS				
Braundmeier-Fleming et al (2016)[32]	18 with IC/BPS and 16 controls	Stool and vaginal swab	16S rRNA gene sequencing	IC/BPS group: - less *Eggerthella sinensis, Colinsella aerofaciens, F. prausnitzii, Odoribacter splanchnicus, Lactonifactor longoviformis*
Abernethy et al (2017)[33]	20 with IC/BPS and 20 controls	Catheterized urine	rDNA sequencing	IC group: - less *Lactobacillus acidophilus*
Nickel et al (2019)[34]	181 with IC/BPS and 182 controls	Midstream urine	Ibis T5000: Multilocus PCR coupled with ESI-TOF-MS	IC/BPS group: - more *Lactobacillus gasseri* - less *Corynebacterium*
Nickel et al (2020)[35]	202 with IC/BPS	Midstream urine	Ibis T5000: Multilocus PCR coupled with ESI-TOF-MS	No overall differences in fungal species/genus composition or diversity by symptom flare status or pain severity. Increased presence and relative abundance of *Candida* and *Malassezia* for high urinary symptoms
Nickel et al (2022)[36]	59 with IC/BPS	Midstream urine	16S rRNA gene sequencing	Male HL group: - more *Negativicoccus succinivorans, Porphyromonas somerae, Mobiluncus curtisii, Corynebacterium renale* No significant species abundance differentiation between overall/female Hunner lesion (HL) and non-HL groups

Table 3
Summary of studies on urolithiasis

Study	Sample size	Material	Analysis technique	Significant findings
Stern et al (2016)[38]	23 with kidney stone and 6 controls	Fecal sample	16S rRNA gene sequencing	Kidney stone group: - more *Bacteroides* - less *Prevotella*
Ticinesi et al (2018)[39]	52 with kidney stone and 48 controls	Fecal sample	16S rRNA gene sequencing and deep shotgun metagenomics sequencing	Kidney stone group - lower fecal microbial diversity - less *Faecalibacterium, Enterobacter, Dorea*
Liu et al (2020)[40]	69 with calcium oxalate kidney stone (43 occasionally, 26 recurrently) and 84 controls	Fecal sample	16S rRNA gene sequencing and deep shotgun metagenomics sequencing	Several SCFAs-producing gut bacteria including *Blautia*, *Anaerostipes*, *Coprococcus*, *Fusobacterium*, *Ruminococcus*, and *Lachnospiraceae* were considerably lower in the gut microbiota among the kidney stone patients compared with the controls.
Kim et al (2022)[41]	183 with kidney stone (97 incidental, 86 prevalent) and 732 controls	Fecal sample	16S rRNA gene sequencing	Nephrolithiasis was associated with a reduced abundance of some key taxa involved in short-chain fatty acid production. Moreover, the abundance of *Bifidobacterium*, which possess oxalate-degrading ability, was higher in the control. No significant difference in the bacterial composition between the incidental and prevalent nephrolithiasis.
Yuan et al (2022)[42]	69 with kidney stone and 84 controls	Fecal sample	16S rRNA gene sequencing	Kidney stone group with high nephrolithiasis risk dietary pattern - more *Pseudomonas, Sphingomonas, Hydrogenoanaerobacterium, Faecalitalea*,

BCa is a disease of older people, with more than 75% being diagnosed at age 65 or older and 45% at age 75 or older.[47] Luzzago et al[48] have reported that a more advanced age is associated with a higher cancer-specific mortality rate in BCa without metastasis. Likewise, elderly over 70 years of age had more *Jonquetella, Proteiniphilum saccharofermentans, and Parvimonas* than the younger group[49] with increased numbers of bacterial genera but decreased the total number of bacteria compared to the younger group. These findings suggest that age-dependent microbiome differences might affect tumorigenesis and aggressiveness of BCa.

Within the last decade, many studies have performed microbiome analyses for BCa group and non-cancer group. These can be broadly classified into studies using urine samples and studies using tissues. Using tissue samples, Liu et al[50] and Parra-Grande et al[45] have analyzed the microbiome of patients with bladder tumor study and found a relative decrease in species by α-diversity in tumor tissues compared to that in non-malignant tissues. Bladder microbiome abundances of phyla such as *Firmicutes* and *Actinobacteria* were similar to urinary microbiome values of previous studies.[51] It has been reported that β -diversity differs between BCa and non-malignant tissue.[52] Recently, Li et al[53] have demonstrated that tumor microbiome correlates with the regulation of epithelial−mesenchymal transition in muscle invasive bladder cancer (MIBC) based on TCGA samples, with significant overlap of genera composition based on 16S rRNA sequencing data.[54] Chen et al[55] have reported that PD-L1-positive cell count is positively correlated with the abundance of the urogenital microbiome.

Using urine samples (urination and catheterization/cystoscopy), some studies have reported significantly greater α and β diversities.[45,56] However, Chipollini et al[57] have demonstrated lower α diversity in BCa compared to matched healthy controls. Wu et al[58] and Zeng et al[59] have shown that richness measures of males with BCa are higher than those of males without cancer. Wu et al[58] have reported that *Acinetobacter*, *Anaerococcus*, and *Sphingobacterium* are increased whereas *Serratia*, *Proteus*, and *Roseomonas* are decreased in 31 male patients with BCa than in 18 age-matched healthy controls. Subsequently, Zeng et al[59] have reported higher microbiome species diversity in a recurrence group than in a non-recurrence group of non-muscle invasive bladder cancer (NMIBC) patients after transurethral resection of bladder tumor (TURBT), with 9 genera increased in the recurrence group. Popović et al[51] have compared 12 patients with BCa and 11 age-matched controls and demonstrated that *Fusobacterium*, *Actinobaculum*, *Facklamia*, and *Campylobacter* genera are significantly enriched in BCa patients. Recently, Ma et al[60] have reported tobacco smoking can change urinary microbial compositions and promote tumorigenesis. Hussein et al[56] have reported microbiome differences between a BCG response group and a BCG-refractory group. They also analyzed differences between BCa and no cancer groups as well as between NMIBC and MIBC. Oresta et al[61] have shown that microbial differences are related to disease progression and that different results of microbiome analysis depend on the type of urinary specimen collection, suggesting that additional research is needed in the future.

To the best of our knowledge, only Mansour et al[54] have compared voided urine and tissue samples from BCa patients and demonstrated no significant change in α diversity, although a significant change in β diversity has been found. Subsequently, they reported that defensins and microbes could affect the development, progression, and treatment options of BCa.[62]

2.5. Microbiome in prostate cancer

Carcinogenic effects of microbiomes on prostate cancer can be largely divided into direct and indirect effects, with the former occurring when microbes directly contact associated tissue and organs, causing pathogenic change. Direct effect of microbiomes occurs quite naturally when commensal microbiota are present, as in the case of colorectal and intestinal malignancies.[63] In contrast, indirect effects are associated with distant causalities such as aberrations in host immunology and systemic inflammation as well as gut absorption of metabolites that can ultimately impact progression into cancer or affect response to treatment when the equilibrium of microbiota is disturbed due to disease or infection.[64] Prostate cancer (PC) has fundamentally been associated with chronic inflammation in tumoral tissues,[65] leading to upregulation of local inflammatory cytokines and subsequent increased risk of malignant transformation.[66] Due to the nature of elevated prostate-specific antigen (PSA) in prostatitis as well as PC, studies of microbiomes in PC can further elucidate the role of bacterial infection in PC pathogenesis and improve the accuracy of PSA as a biomarker for PC[67,68] (Table 5). The proximity of prostate

Table 4
Summary of studies on bladder cancer

Study	Sample size	Material	Analysis technique	Significant findings	Limitations
Parra-Grande et al (2022)[45]	26 paired BCa and adjacent non-tumor mucosa	Tissue	16S rRNA amplicon sequencing	Higher overall richness of microbial composition and increased *Actinobacteria* observed in non-neoplastic bladder mucosa	Cross-sectional design, no true negative control, lifestyle factors not controlled.
Pederzoli et al (2020)[46]	49 therapy-naïve BCa and 59 healthy controls	Urine, Tissue	16S rDNA sequencing	More *Klebsiella* in female BCa urine, with abundant *Burkholderia* identified in BCa tissue in both sexes. Urine microbiome shared >80% of microbiome to tissue.	Life style factors or previous antibiotic treatment not controlled.
Liu et al (2019)[50]	22 BCa and 12 adjacent non-tumor mucosa	Tissue	16S rRNA amplicon sequencing	BCa with lower species richness and diversity, with significant difference in β-diversity. *Cupriavidus* spp., *Acinetobacter*, *Anoxybacillus*, *Escherichia-Shigella*, *Geobacillus*, *Pelomonas*, *Ralstonia*, and *Sphingomonas* enriched in BCa tissue, whereas *Lactobacillus*, *Prevotella_9*, and *Ruminococcaceae* were decreased.	Small sample size, no true negative
Li et al (2021)[53]	405 MIBC TCGA samples	Tissue	Whole transcriptome RNA-sequencing (TCGA legacy database)	Microbes were associated with expression of classical EMT (Epithelial−mesenchymal transition)-associated genes, with abundance related to ECM (extracellular matrix) gene expression. Implicated microbes include *E.coli*, *SM4/1*, and *Oscillatoria*.	Lack strict contamination control.
Chen et al (2022)[55]	28 male NMIBC subjects (9 PD-L1 positive, 19 PD-L1 negative)	Urine, tissue	16S rRNA gene sequencing	PD-L1 positive group had enriched microbiome, with increased *Leptotrichia*, *Roseomonas*, and *Propionibacterium* and decreased *Prevotella* and *Massilia* compared to PD-L1 negative subjects.	Small sample size, midstream urine specimens used
Wu et al (2018)[58]	31 male BCa, 18 healthy control	Urine	16S rRNA amplicon sequencing	β diversity significantly differed between BCa & control, with overall bacterial enrichment in BCa with increased *Acinetobacter*, *Anaerococcus*, and *Sphingobacterium*, and decreased *Serratia*, *Proteus*, and *Roseomonas* genus. *Herbaspirillum*, *Porphyrobacter*, and *Bacteroides* were associated with high risk subgroups.	Small sample size, midstream urine
Zeng et al (2020)[59]	62 male BCa and 18 healthy controls	Urine	16S rRNA amplicon sequencing	Bacterial enrichment in BCa group, with species diversity higher in the recurrence group in NMIBC after TURBT including *Micrococcus* and *Brachybacterium*.	Small sample size, midstream urine
Chipollini et al (2020)[57]	38 BCa and 10 healthy control	Urine	16S rRNA sequencing	Decreased diversity in BCa, with higher species richness and increased *Bacteroides* and *Faecalbacterium*	Small sample size
Popović et al (2018)[51]	12 male BCa age-matched to 11 control	Urine	16S rRNA sequencing	*Fusobacterium* enriched in bladder cancer *Veillonella, Streptococcus* and *Corynebacterium* more in control	Small sample size, midstream urine, only male patients
Ma et al (2021)[60]	15 male BCa, 15 control	Urine	16S rRNA sequencing	α diversity in smokers higher in BCa, with principal component analysis showing higher *Bacteroidaceae*, *Erysipelotrichales, Lachnospiraceae, Bacteroides* in smokers	Small sample size, lifestyle factors not considered
Hussein et al (2021)[56]	43 BCa, 10 control	Urine	16S rRNA amplicon sequencing	β -diversity with *Actinomyces, Achromobacter, Brevibacterium*, and *Brucella* in bladder cancer *Hemophilus, Veillonella* higher in MIBC while *Cupriavidus* higher in NMIBC BCG responded group with more *Serratia* and *Brochothrix, Negativicoccus, Escherichia-Shigella*, and *Pseudomonas* in NMIBC	Small sample size, midstream urine
Oresta et al (2021)[61]	122 BCa, 29 control	Urine	16S rRNA amplicon sequencing	Catheterized urine microbiome increased in bladder cancer exacerbating with disease progression **Catheterized**, bladder washout and midstream showed differed results	Small sample size
Mansour et al (2020)[54]	10 urine and 14 tissue samples from 10 BCa	Urine, tissue	16S rRNA sequencing	*Akkermansia, Bacteroides, Clostridium, Enterobacter* and *Klebsiella*, over-represented in tissue samples than urine.	Small sample size, no negative control
Mansour et al (2022)[62]	55 BCa, 29 BPH	tissue	16S rRNA sequencing	*Staphylococcus*, *Corynebacterium* and *Oxyphotobacteria* genera higher in cancer *Faecalibacterium* and *Bacteroides* genera lower in cancer	

to the genitourinary tract makes it an ideal entity for research due to the high rate of exposure to the indirect microbial pathway from urine and gut as well as direct influence of the tumor environment.[63]

As such, extensive research has been undertaken to determine alterations of microbiota in urine, prostatic fluids, fecal material, plasma, and tissues, showing a generally positive association of PC with certain bacterial species found to be abundant in PC than in benign controls. Analyses of fecal and urine microbiome have provided promising results, identifying bacteria associated with higher GS ($\geq$7 vs. 6) and absolute risk of PC.[69] Identified bacteria include, but are not limited to, *Staphylococcus*, *Streptococcus*, and *Bacteroides*.[70,71] Use of androgen deprivation therapy (ADT) significantly altered microbial diversity, with higher *Ruminococcus gnavus* and *Bacteroides*[72] and decrease in *Corynebacterium*.[73] Matched analyses between hormone-sensitive PC and castration-resistant PC after ADT have shown similar increases of *Bacteroides*, *Fusobacteria*, *Synergistetes*, and *Tenericutes*, with corresponding decreases of *Proteobacteria*, *Actinobacteria*, and *Cyanobacteria*.[74] Use of abiraterone acetate has been consistently correlated with increased an abundance of *Akkermansia muciniphila* in two separate studies.[5,73] However, no difference in β diversity of urine microbiome was identified in a study of a relatively large sample of 135 men,[75] although PC patients had more *Streptococcus anginosus*, *Anaerococcus lactolyticus*, *Anaerococcus obesiensis*, *Actinobaculum schaalii*, *Varibaculum cambriense*, and *Propionimicrobium lymphophilum* than patients in benign groups. However, that study lacked information on whether midstream urine was collected to avoid contaminants. Microbiota in blood are less frequently explored due to a previous perception that blood is immunologically sterile unless infected. However, recent advances in NGS and metagenomics have identified distinct microbial signatures that can distinguish healthy controls from cancer patients.[76] A few studies have also reported differential diversity in prostatic secretions of PC, with no single bacterium identified as a causative factor. Current studies are limited by small sample sizes and the lack of true negative controls in addition to missing comparison with clean-catch urine to remove microbiota of urethral origin.[75,77] No seminal microbiome research has so far accounted for reagent contamination. This may lead to misinterpretation of contaminant microbiome as significant for disease.

Although the indirect pathway of urine or fecal microbiomes has been extensively described in literature, results are inconclusive due to a high risk of contaminant DNA and low microbial biomass in historically "sterile" urine. As such, a handful of studies have examined the local effects of microbiome in radical prostatectomy (RP) specimens in order to identify significant pathogenic microbiomes. Propionibacterium acnes has been implicated as a possible pro-inflammatory bacterial species related to PC.[78] This was supported by an investigation of a Chinese RP cohort ($n = 65$) which found that *Propinibacterium* in addition to *Acinetobacter* and *Pseudomonas* was more abundant in tumor tissues than in nearby benign tissues.[79] A more recent Denmark study on 94 subjects has reported an increase of *Shewanella* but significant decreases of *Bacteroides fragilis*, *Saimirine betaherpesvirus*, *Staphylococcus saprophyticus*, and *Vibro parahaemolyticus*,[80] implying the potential association of specific species with PC pathogenesis. However, these studies utilized formalin-fixed paraffin-embedded (FFPE) samples for analysis, which had a high risk of bacterial contamination, not to mention that *Propionibacterium acnes*, *Pseudomonas*, and *Acinetobacter* are known contaminants in bacterial analysis,[4,8,81] inciting the question of the reliability of such studies that did not sequence negative controls or remove possible contaminating endogenous DNA prior to assessment.

3. Limitations of current research

This narrative review identified discrepancies in both direct and indirect pathways holding some levels of significance. It revealed that some pathogens within the urinary tract might show significantly different diversity and compositions, putatively connecting local and systemic microbial environments to pathogenesis. With the introduction of newer NGS techniques including microbial DNA isolation and purification followed by 16S rRNA amplification and sequencing, it is now possible to perform efficient analysis of the entire genome with an inclusion of relatively larger samples to better represent populational significance. However, 16S rRNA does not differentiate between live and dead bacteria. As such, enhanced quantitative urine culture (EQUC) was introduced to overcome the limitations of routine urine. Compared to conventional methods, EQUC has a greater amount of urine sample on various culture media under different atmospheric conditions for a longer period of 48 hours. Therefore, microbiome detected by metagenomics analysis can better represent live microbiome.[1]

However, 16S rRNA sequencing has several limitations such as the inability to distinguish closely related bacterial taxa, assess viability of microbiota, and link genotypic resistance to a specific organism, not to mention the inherent limitation of 16S rRNA sequencing to accurately report bacterial abundance.[91] Most initial studies have utilized 16S rRNA amplicon sequencing, especially for analyses of gut microbiome. Studies have suggested that genomic DNA can be diluted to less-than-optimal thresholds, introducing a systemic bias that needs to be considered in 16S rRNA sequencing analyses.[92] For example, despite the inclusion of a relatively large sample, Liss et al[70] have used rectal swabs to collect fecal materials, resulting in a low DNA yield. They had to discard 24 out of 133 initial collected cohorts due to extraction failure or poor DNA quality. 16S RNA and DNA sequencing alone cannot detect functionality. Shotgun metagenomic sequencing is needed to fully determine functional annotation.[93] Statistical methods used in interpretation can further affect the final outcome.[94] As described in Salachan et al,[95] the mode of statistical analyses is not always described. It differs between studies. This was exemplified in a study by Ma et al[96] who re-evaluated spatial distribution (inter-subject heterogeneity) using a diversity-area relationship analysis and found that semen microbiome diversity in a populational cohort was not associated with fertility health as previously suggested.[97]

There are also limitations in terms of sampling. Many studies presented above have utilized midstream urine samples. However, such samples could be contaminated by microbiota from the uroepithelium, periurethral gland, or genital tract, leading to improper evaluation of urinary bladder microbiota. Such risk is higher in females. However, there is also a chance of contamination in males from nearby tissues including urethra. Transurethral catheterization could be the most preferred sampling method which could gain similar outcomes less invasively compared to suprapubic aspiration.[98,99]

Current sampling methods for gut microbiome analysis also need to be improved. Fecal sampling via swabs is most commonly used for bacterial flora studies because of its convenience and non-invasiveness. However, the microbiota content of fecal matter is significantly different from that of the lower digestive tract. Other biopsy methods are invasive. They are neither suitable nor ethical to be used as healthy controls. To prevent cross-contamination, intestinal contents should be collected at a fixed point, utilizing less-invasive sampling devices. To meet such requirements, the development of swallowable sampling devices and the introduction of gnotobiotic mice are presented.[100]

Table 5
Summary of studies on prostate cancer depending on sample material

Study	Sample size	Analysis technique	Significant findings	Limitations
Prostate tissue				
Salachan et al (2022)[80]	83 PC and 23 benign from 94 RP specimen, validated in 16 PC and 8 benign	Metatranscriptomics, total RNA sequencing	Significant increase of *Shewanella* and decrease in *Staphylococcus saprophyticus* and *Vibrio parahaemolyticus* in PCa. Over-abudnace of *Microbacterium* species in pT3 tumors vs. T2.	No true normal (used adjacent benign tissue). Contamination not assessed.
Ma et al (2020)[82]	242 PC and 52 adjacent benign tissue RNA sequenced data from TCGA	Whole transcriptome RNA sequencing	*Listeria monocytogenes*, *Methylobacterium radiotolerans JCM 2831*, *Xanthomonas albilineans GPE PC73*, *Bradyrhizobium japonicum* abundantly found in PC but may be associated with anti-tumoral effects	No true normal.
Feng et al (2019)[79]	65 PC RP specimen matched to adjacent tissue	Metagenome and metatranscriptomics	*Escherichia*, *Propionibacterium*, *Acinetobacter* and *Pseudomonas* abundant in prostate, but no difference in PCa and benign tissue, nor based on tumor grade	No true normal
Banerjee et al (2019)[83]	50 PC RP specimen and 15 BPH TURP specimen (all FFPE)	Microarray-based metagenomic and capture-sequencing	High signatures of *Trichinella* in GS $\geq$ 8 and *Astroviridae*, *Borrelia*, *Candida*, *Capillaria*, *Entamoeba*, *Enterobius*, *Histoplasma*, *Legionella*, *Mansonella*, *Porphyromonas*, *Shigella*, *Streptobacillus* in GS 6-7. *Heliobacter* highly associated with low GS and *Dicrocoelium* with T3.	No true normal
Miyake et al (2019)[84]	45 PC RP or biopsy specimen and 33 BPH TURP specimen (FFPE)	PCR screening for 5 bacterial and 2 viral agents	Rate of *Mycoplasma genitalium* infection higher in PC, increasing in extensive pT2c-3b disease.	Limited sample, narrow screening range
Fecal (gastrointestinal) material				
Matsushita et al (2021)[69]	96 PCa and 56 benign, randomized to 114 development and 38 validation cohorts	16S rRNA gene amplicon sequencing	Short-chain fatty acid generating bacteria (*Rikenellaceae*, *Alistipes*, *Lachnospira*) increased in high risk PC (GS $\geq$ 7), with a predictive index generated with 18 bacteria having AUC of 0.85	Limited ethnicity (Japanese), function of microbiota unknown.
Li et al (2021)[72]	56 PC on ADT, 30 PC on RP	16S rRNA gene amplicon sequencing	Low alpha diversity in the ADT cohort, with higher *Ruminococcus gnavus* and *Bacteroides* spp. *Lachnospira* and *Roseburia* were higher in the RP group.	Cross-sectional design, limited sample.
Daisley et al (2020)[73]	68 PC (21 on ADT, 14 on ADT + abiraterone, 33 control)	16S rRNA gene amplicon sequencing	*Corynebacterium* spp. is decreased with ADT, and abiraterone use increases *Akkermansia muciniphila* and modulates patient gut microbiota, potentially influencing treatment response.	Limited sample
Liu et al (2020)[74]	21 at HSPC before ADT, matched with samples recollected after ADT at CRPC	16S rRNA gene amplicon sequencing	Matched compositional analyses between HSPC and CRPC after ADT display increase of *Bacteroidetes*, *Fusobacteria*, *Synergistetes*, *Tenericutes*, as well as decrease in *Proteobacteria*, *Actinobacteria*, and *Cyanobacteria*.	Limited sample, dietary or lifestyle factors not accounted for.
Sfanos et al (2018)[5]	7 mPC, 7 PC with BCR, 7 localized PC, 3 negative biopsy, 6 healthy controls	16S rRNA gene amplicon sequencing	Significant difference noted in alpha diversity between PC and non-PC groups, with increased of *Akkermansia muciniphila* and *Ruminococcaceae* spp. in patients taking oral androgen receptor axis-targeted therapies.	Limited sample, used rectal swabs for collection.
Liss et al (2018)[70]	64 PC and 41 negative biopsy rectal swab samples prior to biopsy	16S rRNA gene amplicon sequencing	Abundant *Bacteroides* and *Streptococcus* spp. in PC, but mostly similar species diversity between groups.	Used rectal swabs for collection.

Urine				
Hurst et al (2022)[85]	46 sequenced samples (24 PC, 22 negative biopsy) with total 318 undergoing urine sediment microscopy	16S rRNA gene amplicon sequencing	*Porphyromonas*, *Varibaculum*, *Peptoniphilus*, and *Fenollaria* spp. newly discovered in PC urine and further associated with poor prognosis (including *Fusobacterium*)	Limited sample, only 16S and RNA-seq evaluation used for urine.
Alanee et al (2019)[71]	14 PC and 16 biopsy negative subjects, each 1 urine and 1 fecal sample prior to biopsy	16S rRNA gene amplicon sequencing	Bacterial clustering in urine drastically different between non-PC and GS 7 but not with GS 6. *Veillonella*, *Streptococcus*, and *Bacteroides* increased in PC, with decrease in *Faecalibaterium*, *Lactobaccili*, and *Acinetobacter*.	Limited samples, urine samples may include prostatic secretion (collected after prostate massage)
Shrestha et al (2018)[75]	Total 135 samples (65 PC, 65 negative biopsy, 5 with initial negative but PC found on later biopsy)	16S rDNA gene sequencing	*Corynebacterium*, *Staphylococcus* and *Streptococcus* most commonly found in all samples, with no difference in β diversity. Identified clusters found more in PC were microbiota implicated in infection.	No true normal
Prostatic and seminal fluids				
Ma et al (2019)[86]	32 PC and 27 non-PC	16S rRNA gene amplicon sequencing	Overall diversity is lower in PC, with significant increase in *Lactococcus*, *Carnobacterium*, and *Streptococcus*, whereas *Cronobacter*, *Alkaliphilus*, and *Paenibacillus* were decreased in PC.	Limited samples, possible contamination from urinary tract.
Chen et al (2015)[87]	6 PC and 6 negative biopsy	Small RNA sequencing to compare miRNA	*Propionibacterium acnes* detected in PC but not in normal samples.	Limited samples, no true normal, 16S rRNA not done. .
Yu et al (2015)[12]	Urine, EPS, seminal secretions collected from 13 PC and 34 BPH subjects	PCR-DDGE with 16S rDNA and qPCR	PC EPS had increased *Bacteroidetes*, *Alphaproteobacteria*, *Firmicutes*, *Lachnospiraceae*, *Propionicimonas*, *Sphingomonas*, and *Ochrobactrum*, with decreased *Eubacterium* and *Defluviicoccus*. *E.coli* was increased in EPS and seminal secretions of PC, whereas *Enterococcus* increased only in seminal fluids.	Limited samples
Serum (plasma)				
Wang et al (2022)[88]	31 PC and 34 health controls	Fungal ITS sequencing	*Filobasidiales*, *Pyronemataceae*, and *Cryptococcus ater* spp. were increased in PC. *Bipolaris* genus, *Sordariomycetesm* and *Phoma herbarum* species were associated with low PSA, high stage, and low risk of relapse, respectively.	Limited samples, only peripheral blood included, cross-sectional design.
Reichard et al (2022)[89]	173 lethal PC and 519 non-lethal PC or never diagnosed male controls	Metabolomics with mass spectrometry	Increased baseline levels of choline, betaine, and phenylacetylglutamine had higher risk of lethal PC.	Non-causal, associative analysis, limited sample without validation.
Poore et al (2020)[76]	59 PC (32 HSPC, 27 CRPC) and 69 non-cancer	Whole transcriptome sequencing	Microbial signatures from cell-free DNA showed significant discrimination of PC from healthy controls.	Limited sample, no true negative
Ou et al (2019)[90]	27 PC undergoing RP, 12 healthy control	cell free bacterial 16S rDNA via qPCR	Similar microbial translocation signatures, but increased 16s rDNA in BCR vs. no BCR and pT3 vs. pT2.	Limited samples

PC, prostate cancer; BPH, benign prostatic hyperplasia; TURP, transurethral resection of prostate; FFPE, formalin-fixed paraffin-embedded, ADT, androgen deprivation therapy; CRPC, castration-resistant prostate cancer; Hormone-sensitive prostate cancer; mPC, metastatic prostate cancer; BCR, biochemical recurrence.

Contamination is a critical issue, where the analysis of low biomass in genitourinary diseases suffers the most. Confounding microbiota can be introduced during sample collection, e.g., midstream "clean catch" urine, or even during handling processing. Thus, blanks should be co-analyzed to exclude both human and technical errors. As such, sample handling in biosafety cabinets and controlled environments should be ideally performed, and should be noted within the methods description. Eisenhofer et al[7] have presented a RIDE checklist to account for cross-contamination and contaminant DNA in blank controls, which includes: a) reporting study design and approaches used to reduce and assess contributions of contamination, b) inclusion of controls to assess contaminant DNA with at least one of each type of negative control, c) determining the level of contamination by comparing biological samples to controls, and d) exploring contaminant taxa and reporting their impact on interpretation. To the best of our knowledge, only two studies in PCa[76,85] have stringently adhered to such guidelines identifying that four new bacteria (*Porphyromonas*, *Varibaculum*, *Peptoniphilus*, and *Fenollaria* spp.) in patient urine with significant association with metastatic disease. Therefore, large-scale, well-controlled studies and meta-analyses are required to accurately evaluate the true influence of microbiomes, which hold much potential as potential biomarkers but is currently inconclusive at best.

4. Conclusion

There is no doubt that inflammation is caused by regional microbiomes in the tumor microenvironment and that chronic infection can influence systemic immunity with the potential use of urinary microbiomes as clinical biomarkers of disease as well as response to treatment and clinical prognosis. However, current research is not without limitations, and no microbiome has been identified as a single causative or definitive factor of pathogenesis. Microbiome research studies in genitourinary disorders and malignancies are still in their infant stages, limited by the lack of control for contaminants as well as the myriad of lifestyles and patient factors as well as confounding factors in current results. Larger, well-controlled trials on urinary tract microbiota are needed to investigate the clinical relevance of urinary microbiota.

Conflict of interest

None of the authors have any conflicts of interest with any institution or product.

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