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Review Article

Microbiology for health care

Unlocking The Human Urobiome: Impact On Health and Disease- A Review

Aishwarya J Ramalingam[1*], Sameena Khan[2], Manonmoney J[3] and Archana R[4]

[1*]*Associate Professor, Department of Microbiology, Sree Balaji Medical College & Hospital, Bharath Institute of Higher Education & Research, Chennai*

[2] *Assistant Professor, Department of Microbiology, Dr. D.Y. Patil Medical College, Hospital & Research Centre, Pune,*

[3]*Associate Professor, Department of Microbiology, SRM Medical College & Research centre, SRM Institute of Science and Technology, Kattankulathur, Chennai*

[4] *Professor, Department of Anatomy, Sree Balaji Medical College & Hospital, Bharath Institute of Higher Education & Research, Chennai*

Abstract: The urinary microbiome or the urobiome are the group of microbes present in the urinary tract. They came into the limelight in the last decade due to advances in diagnostic technologies. Two complementary assays are widely used for research in urobiomes. Next-generation sequencing (NGS) of 16S ribosomal RNA (rRNA) paired with enhanced urine culture techniques (EUCT) are widely employed for research. This has paved the way for investigations on sterile body sites such as urine thereby breaking the myth of urine being considered sterile. EUCT, such as expanded quantitative urine culture (EQUC) contributed to the evidence that the microbes detected by the NGS are still alive. EQUC has been employed in clinical laboratories since the last decade and is indicated only when there are unexplained clinical symptoms and conventional urine culture is negative. Our aim is to have a comprehensive review study on the urobiome concerning health, its association with urological pathologies. Our main objective is to collect review and research articles using databases and review them for obtaining in-depth knowledge of the urobiome as well as to identify possible alternate study areas. The urobiome is a new area with minimal information available. This review helps the researcher to comprehend this upcoming area easily. Investigations into urobiome research, however, could have a considerable impact on our understanding of the pathogenesis of urogenital disorders and even reveal novel possibilities. Thus, the forthcoming years will open a ripe ground for future research into diagnosis, treatment and prevention strategies for urological pathologies.

Keywords: Human Microbiome, Microbiota, Urobiome, 16S rRNA Sequencing, Dysbiosis, Viromes and Bacteriophages

***Corresponding Author**

Aishwarya J Ramalingam , Associate Professor, Department of Microbiology, Sree Balaji Medical College & Hospital, Bharath Institute of Higher Education & Research, Chennai

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1. INTRODUCTION

The term "microbiota" refers to the entire set of microorganisms found in a definite biologic niche.[1] Present evidence states that each human cell is associated with approximately ten bacterial cells forming a giant ecosystem thereby contributing towards the physiological functions of the human body such as vitamin K production, its role in clotting mechanism and also to the development of immunity.[2] Human microbiota is composed of virus, fungi, protozoa, archaea in addition to bacteria. It is postulated that there might be a competition between the microorganisms and/or their products and host cells; between microorganisms maintaining a dynamic state of equilibrium. This hypothesis has led to the emergence of research on the microbiota with the help of newer diagnostic methods. Next-generation sequencing (NGS), a recent invention, has proved its efficacy in genomic level by targeting entire genomes. Metagenomics, a branch of NGS-based research aims at sequencing highly variable fragments encoding 16S rRNA which enables identification of bacteria without the need for culture.[3,4] Subsequently, this has led to the emergence of the term "microbiome", which refers to the entire set of genomes from all organisms in the environment. The Human Microbiome Project (HMP) which aimed at particular body sites such as gastrointestinal tract, oral cavity, skin and subsequently urine which were considered to be sterile.[5] Thus, NGS technology has made it possible for researchers to investigate and comprehend the urobiome from a wider and more in-depth standpoint. Urinary tract infection (UTI) is one of the most common healthcare-associated infections and its epidemiology reflects overall drug resistant pattern in certain hospital settings. The diagnosis of UTI are based on urine culture on specified media such as MacConkey and blood agar with a significant colony count of more than or equal to 10^5 colony-forming units (CFU)/mL (example, UTI caused by fast-growing uropathogenic *Escherichia coli*). Clinical presentations such as increased urine frequency, dysuria associated with a negative urine culture report is the most important challenge faced by clinicians. In order to achieve promising outcomes with respect to microbiology culture reports, enhanced urine culture techniques (EUCT) was proposed. It includes increasing the urine volume for culture, widen the selection of culture media, increasing the time for bacterial culture (especially facilitates slow-growing bacteria). Though its practical implementation has not yet been thoroughly achieved universally.[2,6] Until lately, urine was assumed to be a sterile body fluid which was declared unsterile only because of infection.[7,8] With the rise of NGS-based metagenomic studies, there has been a fall in the belief of urine sterility as it reveals that microbial community is present in pathological condition as well as in asymptomatic individuals. NGS based studies show that the urinary tract harbours different types of microbes in healthy people and their alteration in its composition affects the health status of the urinary tract.[8,9] The microbial community of the urinary tract is called the urobiome which comprises of bacteria and viruses such as eukaryotic viruses or bacteriophages.[10] Majority of the research work on microbiome were on the gut microbiome and its metabolites however urobiome is receiving more attention. Currently, research activity is directed towards urobiome to understand its significance with regard to chronic kidney disease, post-kidney transplant. It is generally known that there is a bacterial community in the urinary system, both in symptomatic and asymptomatic individuals. Large alterations in the urinary bacterial communities are observed in some chronic diseases, such as interstitial cystitis/painful bladder syndrome and genitourinary diseases, showing that the local microbial populations change throughout disease conditions. An increasing amount of research refers to the existence of numerous microorganisms in the bladder and kidneys of not only symptomatic but asymptomatic individuals as well. Urological illnesses such as urinary tract infections (UTI), urolithiasis, and others are caused by a number of pathogenic bacteria, whose pathophysiology is now well known. In addition to the maintenance of urinary health, urinary microbiomes also have a role in the development of a number of infectious diseases, cancer, and even some urological disorders.[11] However, by intensifying our efforts to comprehend the urine microbiome, the precise function of urinary microbes has to be further defined. Large alterations in the urinary bacterial communities are observed in some chronic diseases, such as interstitial cystitis/painful bladder syndrome and genitourinary diseases, showing that the local microbial populations change throughout disease conditions.[12] Thus, this review article focusses on the various studies undertaken to characterise the components of urobiome, various difficulties and prospects originating from these studies.

2. BACTERIA OF THE URINARY TRACT

2.1. Bacteria in The Urobiome and Its Association with Health

Metagenomic approach not only provides enormous qualitative information regarding the phylum, genus, species identification of the microorganism but also the quantitative data about the diversity of microorganisms and its contribution to the microbiome. It is well established that the type of urine specimen collection influences the interpretation of results. Though clean-catch midstream urine is collected for urine culture and other diagnostic methods, in females, it should be described as genitourinary specimens as the sample collected is contaminated with vulvovaginal microbes. In contrast, sample collected by transurethral catheterisation and suprapubic aspiration are described as bladder specimens.[13,14] Hence, standardisation of specimen collection techniques, specimen preservation methods and analytical approaches are the need of the hour. Research groups focusses on female bladder urobiome. The biomass of urobiome is relatively less than vaginal microbiome and is composed of *Lactobacillus, Gardnerella, Streptococcus*. A proposal at "The Prevention of Lower Urinary Tract Symptoms (LUTS) Consortium"[15] defined bladder health as "A complete state of physical, mental, and social wellbeing related to bladder function and not merely the absence of LUTS. Healthy bladder function permits daily activities, adapts to short-term physical or environmental stressors, and allows optimal well-being (travel, exercise, social, occupational, or other activities)." This proposal of bladder health definition may contribute to improvements in clinical phenotyping and associated urobiome status. Prior investigations in individuals without symptoms as an indicator of normalcy have revealed relationship between female bladder urobiome and post-catheterisation UTI.[16] Gottschick *et al.* detected eight urotypes (UT) from UT1 to UT8 in women with bacterial vaginosis. UT1 comprises of *Prevotella amnii, Sneathia amnii, Gardnerella vaginalis,* and *Atopobium vaginae.* UT2- *Lactobacillus iners,* UT3- Enterobacteriaceae, UT4- *Enterococcus faecalis,* UT5- *Streptococcus agalactiae,* UT6- *Citrobacter murliniae*, UT7- *Lactobacillus crispatus,* UT8- no dominant organism. This was classified based on the predominance of a particular organism.

bacterial vaginosis. UT1-UT6 were present in asymptomatic and symptomatic women whereas UT7 was found in healthy individuals. UT2 and UT7 were identified exclusively in women.[17] The major drawback is the limited cases in each study, making it another challenge to compare various races and geographical locations. Various studies showed the increased diversity of urobiomes in females compared to males. Fouts *et al*. identified urotypes dominated by Lactobacillus in females and by Gram-positive bacteria in males.[18] In another study, *Lactobacillus, Gardnerella, Staphylococcus, Corynebacterium, Streptococcus* were the dominant organisms isolated in females.[19] The findings by Lewis *et al.*[20] were consistent with the earlier mentioned studies concerning increased diversity of female urobiome, but the method employed for investigation was pyrosequencing in asymptomatic individuals. Surprisingly, there are many studies on gender differences in the urotypes. These are summarised in Figure 1.

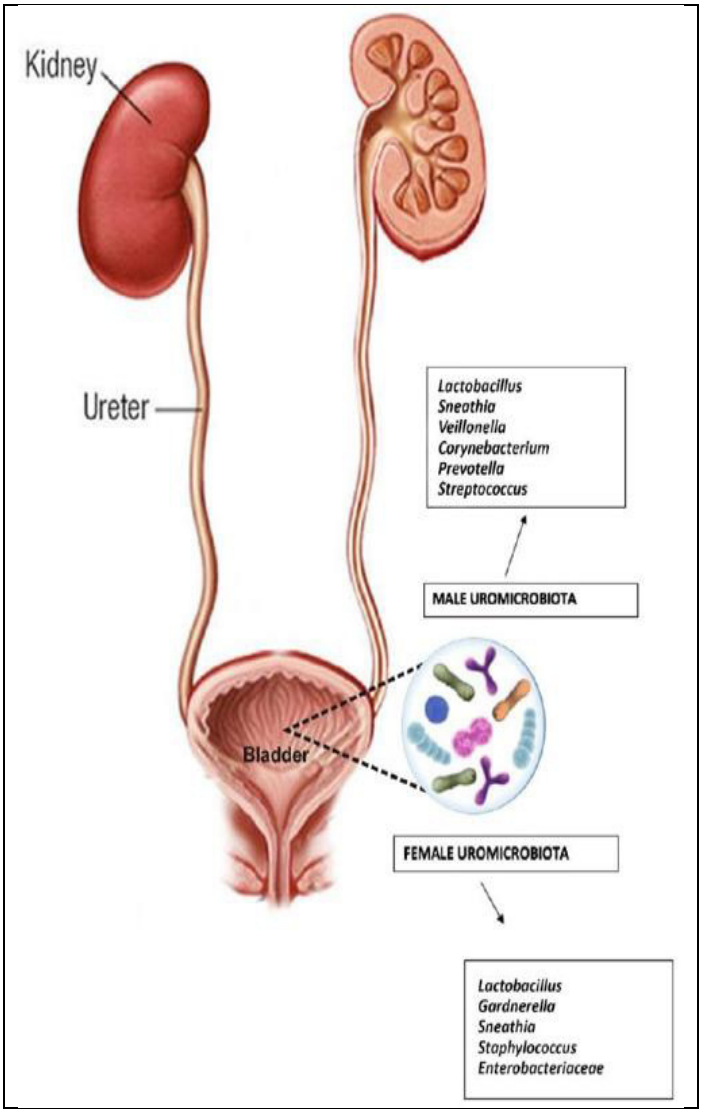

Fig1: Gender-wise urobiome composition in healthy individuals based on 16S rRNA sequencing[21]

It was found that in females between the age group of 20 and 49, organisms isolated belonged to *Gardnerella, Neisseria, Rhodopila, Azospira, Coriobacterium, Sutterella, Tepidomonas* whereas between the age group of 50 and 70, organisms belonged to *Peptostreptococcus, Sneathia, Brevibacterium, Catonella, Methylovirgula, Thermoleophilum, Caulobacter*. In males between the age group of 20 and 70, *Lactobacillus, Pseudomonas, Actinobaculum* were isolated.[21] However, newer genera isolated from individuals over 70 years of age are represented in table 1. The common organisms in males (>70 years) isolated were *Lactobacillus, Corynebacterium, Fusobacterium, Mobiluncus, Aminobacterium, Anaerococcus, Campylobacter, Eubacterium, Finegoldia, Mycoplasma, Porphyromonas, Peptococcus, Peptostreptococcus, Prevotella, Rikenella, Saccharofermentans.*

Table 1: Newer genera contributing to the urobiome composition in healthy individuals above 70 years based on pyrosequencing[21]

Female	Male
Actinomyces, Saccharofermentans, Arthrobacter, Gulosibacter, Jonquetella, Modestobacter, Oligella, Parvimonas, Rhodococcus, Proteiniphilum	*Atopobium, Parvimonas, Atopostipes, Anaerophaga, Anaerosphaera, Actinobaculum, Azospira, Butyricicoccus, Catonella, Dialister, Filifactor, Microvirgula, Peptoniphilus, Proteiniphilum, Pseudoramibacter, Rikenella, Sediminitomix, Saccharofermentans,*

2.1.1. Gut Microbiomes and Urinary Tract

There are studies which support the evidence that the gut microbiomes influence the health of distant body sites. Similarly, there are evidences indicating the relationship between gut microbiomes and the kidney thereby aiding the gut-kidney axis.[22] Any gut microbiome dysbiosis affects the health of the urinary tract thereby contributing to chronic kidney disease, urinary tract infections, urinary stones.[23]

2.1.2. Vaginal Microbiomes and Urinary Tract

The lactobacilli such as *Lactobacillus iners*, *L. crispatus, L. gasseri, L. jensenii* are predominantly found in the vagina of women in reproductive age group. They produce bacteriocins, hydrogen peroxide and lactic acid. Enormous production of lactic acid thereby contributes to the low pH of vagina.[24] This confirms the protective mechanism of vaginal lactobacilli. In some women of reproductive age, low levels or even complete absence of lactobacilli are found but its function is overcome by other organisms such as *Actinobacteria, Firmicutes* but the level of protection by these organisms is relatively lesser compared to women harbouring *Lactobacilli*.[25] Dysbiosis of the vaginal microbiome can cause bacterial vaginosis (BV).[26]

2.2. Bacteria in The Urobiome and Its Association with Disease

There are wide studies about urobiome and its relation to urological pathologies such as bladder cancer, sexually transmitted infection (STI), urinary incontinence, kidney stones, UTI, chronic kidney disease.[27] Mulder *et al*. found that prolonged treatment with antibiotics decreased the prevalence of *Lactobacillus, Finegoldia.*[28] However, it is still unclear whether the urobiome imbalance existed prior to infection or whether it is an adverse effect of prolonged antibiotic therapy thereby resulting in infection. Urobiome and its connection with bladder pathologies have remained a field of interest for researchers. It is found that there has been considerable difference in bladder microbiome between the bladder cancer patients and healthy individuals. Studies by Wu, P *et al*. and Bu˘cevi´c Popovi´c *et al*. detected *Fusobacterium* species which was found to be protumorigenic whereas bacteria belonging to the genera *Bacteroides*, *Porphyrobacter, Herbaspirillum* denoted prognosis with respect to progression and recurrence.[29,30] *Streptococcus, Veillonella* and *Corynebacterium* were the most common in urine of healthy individuals[30]. Nelson *et al.* studied the relationship between urine microbiomes and asymptomatic STI in males. It was found that patients with STI had abundant anaerobic bacteria.[31] Gottschick *et al*. compared the urobiomes of women treated for bacterial vaginosis with metronidazole with those of healthy individuals.[17] The above-mentioned studies failed to express the diversity among samples. In contrast, a study by Mueller *et al.* indicated the increasing levels of diversity, which was extremely reduced in patients with bacterial vaginosis on treatment with metronidazole, decreased in bacterial vaginosis and very significant in health.[16] A multicentric cross-sectional study was performed by the NIH-NICHD-funded Pelvic Floor Disorders Network using 16S rRNA sequencing on catheterised urine samples of well-characterised 84 asymptomatic women and 123 women with mixed urinary incontinence (MUI). The age of the participants was dichotomised at 51 (median age for menopause). The urobiome characteristics were analysed using Dirichlet Multinomial Mixture modelling. Based on >50% microbial dominance, the researchers detected six bacterial communities. It is suggested that *Lactobacillus* spp. might be associated with MUI.[32]

2.2.1. Bacteriology of Kidney Stones

Although numerous microbes have been associated with infected urolithiasis, 10-15 % encountered are by urease-producing organisms like Proteus spp. The others include *Pseudomonas*, *Klebsiella*, *Providencia*, *Serratia spp*., and *Staphylococci.* The presence of this microbiome can impact stone formation directly or indirectly. Alteration in the host environment favours the precipitation of calcium and magnesium ions to create struvite and apatite stones rather than direct microbial by-products. Nanobacteria often referred to as calcifying nanoparticles (CNPs), are known to accelerate the formation of calcium phosphate from the bloodstream. These CNPs have been found in various morbid conditions of the cardiovascular system and kidneys. The chance of bacteria sticking to crystals and forming aggregations leading to endothelial damage and later calcification may be one reason behind this.[33] Also, the ammonia produced by these reactions is also incorporated into the resulting stone. Recent studies based on conventional and genetic identification have found bacteria to be associated with urinary stones, which proposes that the resident microbiota present in the upper urinary system contributes to formation of calculi. In addition, active dissolving and recalcification sections have indeed been found in recent biogeochemical studies on kidney stones, which are consistent of biofilm production.[33,34]

2.2.2. UTI

Urinary tract infections remain as the commonest bacterial infections and is the major source for Gram negative bacteraemia leading to urosepsis. Some Gram-positive cocci are inhabitants of the lower gut and female reproductive system. *Staphylococcus saprophyticus* which resides in the gut, perineum and female genital tract is one of most common causes of UTI. CAUTI is associated with *E. faecalis* and *E. faecium*. Group B *Streptococcus* (GBS) causing UTI is predominantly found in pregnant, diabetic, elderly and immunocompromised patients.[35]

2.2.2.1. Gut Microbial Dysbiosis in UTI

The initial steps in pathogenesis of UTI includes contamination and colonisation of periurethral space and the urethra by gut microbiomes; and finally it ascends to the bladder.[12] Eighty percent of community acquired UTIs are caused by uropathogenic *E. coli* (UPEC) whereas healthcare associated UTIs are caused by *Staphylococcus, Klebsiella, Proteus, Enterococcus, Enterobacter.*[12,36] UPEC strains causing UTIs are similar to that found in the gut thus proving their origin however they differ from commensal *E.coli* by the presence of certain virulence factors such as surface polysaccharides, adhesins, toxins[37]. A study by Thänert *et al*. found that there is frequent dissemination of uropathogens from the gut to the urinary tract in patients with recurrent UTI (rUTI).[38]

2.2.2.2. Vaginal Microbial Dysbiosis in UTI

It is found that certain uropathogens such as *E. coli* colonise the vagina contributing to rUTIs in women.[39] There are certain fastidious organisms which are under-reported and some uropathogens which are under-appreciated. It is found that

Gardnerella vaginalis is associated with rUTIs, pyelonephritis.[17] Some vaginal microbiomes can initiate a disturbance in the host-pathogen relation thereby causing immunomodulation and triggering injury during their brief stay in the urinary tract. This is called "covert pathogenesis".[40] This hypothesis was demonstrated in lab mouse which proved that Group B *Streptococcus* (*S. agalactiae*) in the urinary bladder facilitated the survival of *E.coli* during its initial stages of pathogenesis, regardless of the host-immune response.[41] Another study on animal models demonstrated that *G. vaginalis* triggered rUTI.[42] In post-menopausal women, decrease in estrogen causes alteration in vaginal microbiome which is characterised by decrease in *Lactobacillus* spp., vulvovaginal atrophy leading to UTI and rUTI. This constitutes the genitourinary syndrome of post-menopausal women due to changes in the hormone levels.[43] There are studies which found that women with BV are associated with a higher risk of UTI.[41] Unfortunately, the exact mechanism is still in its infancy. It is believed that *Lactobacilli* which produce lactic acid, hydrogen peroxide creates an unfriendly environment for uropathogens.[44] Many studies provide compelling evidence that there were enormous anaerobes in the urine of women with rUTI compared to healthy controls.[45] In such patients, administration of probiotics vaginally has been found useful.[46] All the above studies indicate an association between vaginal and urinary bladder dysbiosis.

2.2.2.3. Gram Positive Cocci and UTI

One of the risk factors for UTI due to *S.saprophyticus* is sexual intercourse.[47] *S.saprophyticus* UTI in males are detected in elderly, immunocompromised patients. The symptoms are more severe than UTI caused by *E.coli* and can even lead to complications such as acute pyelonephritis.[48] Methicillin resistance in *S.saprophyticus* (less than 8%) encoded by *mecA* gene on staphylococcal-cassette chromosome (SCC) is highly uncommon.[49] One of the important virulence factors which promote adherence and colonization of *S.saprophyticus* are the adhesins. They include Aas[50], Ssp[50,51], cell wall-attached surface proteins, SssF, Uro-adherence factor A (UafA)[52] and UafB. Aas and UafA are haemagglutinins; Ssp is a lipase; SssF is plasmid-encoded and confers resistance to linoleic acid.[53] The other virulence factors include capsule and enzymes such as sortase[54], urease, D-serine deaminase. The enzyme urease facilitates formation of kidney stones[55] and D-serine deaminase facilitates survival of *S. saprophyticus* in urine containing elevated D-serine.[56] *S.aureus* is most commonly associated with CAUTI and isolated from pregnant women.[57] Majority of them are methicillin resistant. The most important virulence factor of *S.aureus* is production of the enzyme urease.[58] *S.epidermidis* which is a Coagulase negative Staphylococci and a commensal of the human skin is associated with biofilm on indwelling urinary catheters causing CAUTI (2.5%) and majority of them are methicillin resistant. Although *E. faecalis* and *E. faecium* are the third most common cause of hospital acquired UTI, they contribute only to an average of about 20% of community acquired UTI.[59] There are studies which show increased incidence of enterococcal UTI in diabetics whereas in some studies there was no significant increase in diabetics compared to non-diabetics.[60,61] Vancomycin resistant strains particularly among *E. faecium* pose a potential threat with regard to treatment. One of the virulence factors of *E.faecalis* are its ability to adhere to urinary catheters and form biofilms. Esp, *E. faecalis* surface protein is found to promote *in vitro* biofilm formation.[62] It is found that the endocarditis and biofilm-associated pilus (Ebp) and Sortase A (SrtA) promote in vivo biofilm formation in patients with CAUTI.[63] The other virulence factors are enterococcal fibronectin-binding protein (EfbA), collagen adhesin (Ace). [64] *Streptococcus agalactiae* or GBS, is a commensal found in the vagina and lower gastrointestinal tract. It causes nearly two percent of UTI mainly in elderly, diabetics, immunocompromised patients and pregnant women. It can lead to complications such as pyelonephritis and urosepsis especially in patients with pre-existing kidney and bladder abnormalities.[65] GBS bacteriuria in pregnancy can lead to neonatal meningitis and sepsis.[66] Hence CDC suggests thorough screening of pregnant women and administration of prophylactic antibiotics during delivery.[67] As studies on animal models are limited, information on the virulence factors of GBS are also limited. However, some studies in mice showed that the presence of sialic acid in the capsular polysaccharide contributes to the virulence. There is also enormous production of interleukins such as IL-1α , IL-9, IL-10.[68]

2.2.2.4. Catheter-Associated UTI

The etiology of catheter-associated UTI (CAUTI) is found to be the patient's gut microbiome and is associated with in-dwelling urinary catheters.[69] These organisms have the potency to form biofilms and are highly resistant to antimicrobial therapy which can result in acute pyelonephritis, urosepsis and eventually death. The most common organisms (polymicrobial communities) associated with CAUTI are *E.coli*, *Enterococcus faecalis, E. faecium, Enterobacter* spp., *Pseudomonas* spp., *Klebsiella* spp.[70]

2.2.2.5. Lab Models for UTI

The host response to UTI caused by *Staphylococci* are well explained in animal models. It is found that transurethral inoculation of *S.saprophyticus* in mouse bladder resulted in hundred times more yield of the organism from the kidneys compared to the bladder which lasted for about 14 days post-infection suggesting its predilection to cause pyelonephritis. It was found that there was increase in pro-inflammatory cytokines, enhanced macrophage and neutrophil infiltration in the kidneys which spiked at 2 days. The immune response to UTI due to *E. faecalis* are similar to *Staphylococci.* This was observed in murine model which demonstrated kidney tropism.[71] The organism survives within the human neutrophils and macrophages. The oxidative stress mechanism, Ace, extracellular polysaccharide contributes to survival within macrophages.[72] In case of *E.faecium,* TLR-signaling plays an important role in neutrophil infiltration in murine model.[73] Polymicrobial UTIs are common in immunocompromised, elderly and in patients with long-standing urinary catheters. Since the major sources for UTI are considered to be patients' own gut and vaginal microbiome, several lab models have been employed by investigators to study the effect of these organisms in the urinary tract of model lab animals. Synergism between organisms such as *Staphylococcus saprophyticus, Proteus mirabilis* were demonstrated by transurethral inoculation in a rat model.[74] Synergism between *P. mirabilis* and UPEC was also studied in these animals which together yielded greater colony forming unit (CFU) than monomicrobial infection.[75] Also, urolithiasis was identified in murine model inoculated with both *P. mirabilis* and *Providencia stuartii.*[76] Pyelonephritis caused by *P. aeruginosa* and aggravated by *E. faecalis* was demonstrated in mice model.[77] Immune modulation caused by GBS affects the host-immune response thereby leading to severe UTI by UPEC.[78] Our current

knowledge on the response of the immune system to pathogens and commensals are limited. However, there are various researches in this field. The gut barrier plays an important role in innate immunity. The other factors which contribute to membrane impermeability include autonomic nervous system, defensins, Toll-like receptors (TLR). All these facilitate gut homeostasis. TLRs detect microbe-associated molecular patterns (MAMPs) of the microbes. MAMPs are capsular polysaccharides, lipopolysaccharide (LPS), unmethylated bacterial DNA, flagellin. On stimulation of TLRs, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κ B) is released which further releases cytokines, acute phase reactants.[79]

2.2.2.6. Biofilms and UTI

In chronic urinary tract infections, the bacteria persist for longer time and forms bacterial microcolonies sporadically. These microcolonies have the highest capacity to adhere to the epithelium of the ductal system leading to continuous immune stimulation and subsequently chronic inflammation sets in. when the patient is on antibacterial therapy the antibiotics cannot eradicate these adherent microcolonies or the adherent bacteria. Uropathogenic strains can maintain high titres in the urinary tract for several days and can have type - 1 fimbria mediated invasion evading the innate defences. The clusters of microcolonies become compact and get organised in to biofilm like structures termed as the intracellular bacterial communities(IBC). These IBCs can create a chronic bacterial reservoir which can persists for several months. This mechanism increases the invasion process infecting the entire urinary tract and the chronicity or the recurrence /relapse.[80] Biofilm can be formed on foreign bodies in the urinary tract like indwelling urethral catheters. Urinary catheters are targets of biofilm development on their inner and outer surfaces once they are inserted. A frequent clinical problem with the use of medical biomaterials in the urinary tract is the development of encrustation. Progression of these encrustations eventually blocks the catheter lumen. Biofilm formation and encrustation also contribute to the reduced efficacy of antimicrobial catheter coatings. Most common biofilm forming bacterial agents in urinary tract are *Proteus, Staphylococcus, op–Pseudomonas and Enterobactericiae*. There are various methods to prevent biofilm formation including the antibiotic impreganated catheters, nanostructural particles coated antibiotics, Hydrophilic-coated catheters, antiseptics coated catheters and use of low-energy surface acoustic waves (SAW) [80]. Multiple virulence factors aid the pathogenicity in the uropathogens. One such potential virulence determinant is the ability to form biofilm in biotic and abiotic surfaces. Bacterial biofilms are responsible for resistant infections and 80-90 percent of for 40-50% 0f Hospital Acquired Infections.[81] Biofilm formation in the Indwelling catheter is a common phenomenon and a virulence determinant involved with chronic, persistent and refractory urinary tract infections.[82,83] Among the uropathogens, biofilm formation is exclusively associate with quorum sensing phenomenon and bacterial cell to cell communication and coordinating the multiple virulence factors. The antibacterial efficacy of the antibiotics is reduced in the bacterial biofilm infection due to the inefficient drug delivery to the nidus. Biofilm forming isolates are entirely different from their bacterial community with regard to the genes that are transcribed.[84] Biofilms is a significant threat for patients requiring indwelling medical devices since the treatment of bacteria associated with biofilms are challenging with conventional antimicrobial therapy. Considering the urinary catheterisation, as an inflammatory response fibrinogen is released and gets accumulated in the bladder as well on catheters. The fibrinogen acts as growth promoter for the bacteria and also helps in the biofilm formation.[85] The various mechanisms for tolerance to antibiotics by bacterial biofilm are poor antibiotic diffusion through biofilm matrix, transmission of the resistant gene among the bacterial community ,efflux pump expressions and changes in pH values, reduced metabolism and growth rates, presence of dormant or persister cells, induction of a biofilm phenotype.[86,87] With the improving knowledge about the biofilm there are novel antibiofilm approaches available now. The antibacterial potential of nanostructural metal ions is one of the recent approaches. The nanotechnology is also used as an efficient drug delivery system in combating the resistance of bacterial biofilms. Antimicrobial coating with nanoparticles, combinations of enzyme inhibitors and bacteriophages has increased the efficacy of the antimicrobial agents up to 99 % especially in the device associated infections. Recent trials involve the quorum sensing inhibitors which prevents biofilm formation through modulating the quorum sensing phenotypes.[88]

2.2.2.7. Emerging Gram-Positive and Polymicrobial Causes of UTI

Many genera such as *Actinobaculum, Actinomyces, Atopobium, Bifidobacterium, Corynebacterium* in addition to *Gardnerella* have been detected as part of urobiome. Thus, future research are desired to study the prospective role of these organisms in UTI as they are often under-diagnosed or dismissed as microbial contamination.[35] A study was done by Fok *et al*.[89] using 16S rRNA sequencing on catheterised urine, vaginal and perineal swab samples of 126 adult women. The samples were taken prior to the urogynaecological surgery. *Lactobacillus* was the predominant organism isolated from bladder urine and vagina which were 30% and 26% respectively. Two fastidious anaerobic Gram positive uropathogens- *Finegoldia magna* and *Atopobium vaginae* were detected and they were not influenced by age or hormone state. The latter organism is commonly detected in the vagina of women without bacterial vaginosis and it is still unclear whether all the strains of *A.vaginae* cause symptoms. They are emerging uropathogens and clinicians are unfamiliar with these uropathogens. This study suggests the need for preoperative evaluation of the urobiome in order to reduce the perioperative risk of UTI. In addition to 16S rRNA sequencing, enhanced urine culture techniques have enabled researchers to set a reference of bladder-specific microbes to analyse the contributions of these urobiomes in health and disease. *Aerococcus* is a Gram-positive cocci, facultative anaerobe which are isolated from urinary tract and vagina. *A.sanguinicola, A.viridans, A.urinae* causes UTI leading to urosepsis in patients with underlying urological pathologies. It is also found that *A.urinae* is resistant to sulphonamides.[90] *Corynebacterium urealyticum* is a Gram-positive bacilli, facultative anaerobe and are commensals of the skin. They cause pyelitis, alkaline-encrusting cystitis with struvite deposition.[91] Various studies showed resistance to multiple drugs such as ciprofloxacin, gentamicin, ampicillin, imipenem[92]. *Actinobaculum, Arcanobacterium, Mobiluncus, Actinomyces* belong to the family *Actinomycetacea*.[93] They are facultative anaerobes. *Actinobaculum suis, A. schaalii* cause UTI. *A. schaalii* is a short, Gram-positive bacilli causing UTI in elderly patients with prior urological pathologies such as CKD.[94] *Gardnerella vaginalis* which are normal commensals of the vagina has been associated with UTI in addition to BV. They are mostly

polymicrobial with underlying urological pathologies.[95,96] It was found that aerobic culture yielded a single organism (*E.coli*) but another culture-independent method which was run in parallel yielded multiple organisms (*Aerococcus, Actinobaculum)*.[14] Hence, further research is essential to differentiate the polymicrobial causes of UTI or "Contamination" with Urinary Microbiota. All these organisms are underreported because they require fastidious conditions for their growth making it difficult for isolation and identification. They are overlooked as contaminants because certain resident flora share similar colony appearance making it difficult to interpret them as potential pathogens.[97] Also, lack of standard detection techniques have contributed to the same. In order to establish these organisms as uropathogens, extensive experimental animal studies are essential. Few labs encourage use of both selective and non-selective agar; increasing the incubation period; employing Giemsa stain in addition to Gram stain of urine for enhancing the interpretation of urine culture reports.

2.2.2.8. Non-Surgical Management of UTI

The management of UTI (Acute/chronic/ recurrent or relapse) is multimodal. The mainstay and first line of management is the antimicrobials during the active infection. But the long term management mainly focuses on prevention of relapse and recurrence. The non-specific measures like increase of fluid intake, sexual hygiene, reduction of post-voidal residual urine volume, pelvic floor exercises, hormonal supplements (oestrogen in post-menopausal women), probiotics and diet modification can help in the reduction of severity. The non-surgical management of UTI can be as two categories: antimicrobial dependent and non-antimicrobial treatments. Antimicrobial dependent treatment involves the prophylactic antibiotics give as a combination of drugs for longer duration and in low dose minimising the adverse effects of the drugs itself. The other way is the delivery mode modifications like direct instillation of antibiotics or instillation of hyaluronic acid and chondroitin sulphate intravesically. Non-antimicrobial treatment methods are more recent and has future perspective in treating the recurrent or resistant urinary tract infections. The application of antibodies therapy, phage therapy, immune stimulation, usage of bacterial lysins, peptides and vaccines (combination of killed uropathogenic strains) which are more promising in the treatment of recurrent/relapse/resistant UTIs.[98,99] (Figure 2).

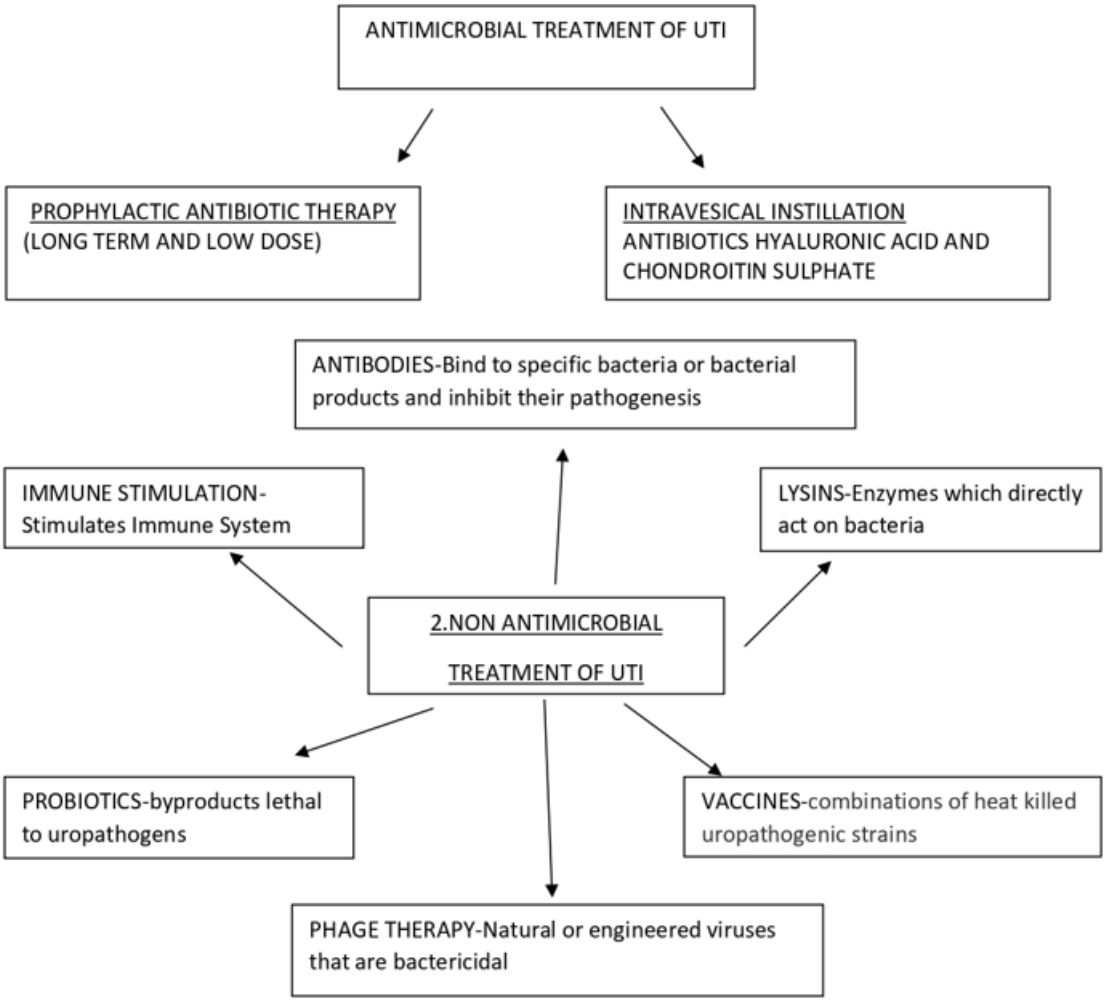

Fig 2: *Non-surgical treatment of UTI*

2.2.3. Role of Urobiome in Chronic Kidney Disease

Chronic kidney disease (CKD) is complex and hence it is challenging to investigate and establish association between urobiome and kidney pathologies as the studies on this area are even more limited. In patients with CKD, the diversity of microbes in the urinary tract is related to the eGFR value. This was supported by Kramer *et al*. in their study on patients with CKD stages 3 to 5. There was significant diversity of urobiomes; the urobiome was less diverse in stage 5 and highly developed in stage 3.[100] (Table 2)

Table 2: Bacteria in the Urobiome isolated from different stages of CKD	
CKD stage	**Bacteria isolated**
Stage 3–5	*Gardnerella, Staphylococcus, Bifidobacterium, Lactobacillus, Enterobacteriaceae, Streptococcus, Corynebacterium, Anaerococcus, Prevotella, Aerococcus*

2.2.3.1. Gut Microbial Dysbiosis in CKD

Dysbiosis in the gut alters the permeability of intestinal mucosa releasing pro-inflammatory mediators and endotoxins into the blood thereby initiating inflammatory cascade in chronic kidney disease.[101] Bacteria such as *Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria* constitute the gut microbiome.[102] There are studies by Di Iorio *et al* and Wang X *et al* which established that *Bifidobacterium* and *Lactobacilli* were negatively correlated with progression in CKD.[103,104] Another study found negative correlation between uraemic toxin accumulation and disease progression, in the presence of urobiomes such as *Prevotella, Roseburia* and *Faecalibacterium prausnitzii*. However, in this study there was positive correlation between accumulation of uraemic toxins in 223 patients with chronic kidney disease and presence of urobiomes such as *Fusobacterium nucleatum, Eggerthella lenta, Alistipes shahii.*[104] Wu I-W et al. in a study on 92 patients with chronic kidney disease found that there was enormous *Paraprevotella, Pseudobutyrivibrio, and Collinsella stercoris.*[105] Hence, it is found that the gut microbiomes have a crucial effect on CKD outcomes.

2.2.4. Urobiome and Kidney Transplant Recipients

In kidney transplant recipients, the urobiome undergoes certain changes thereby resulting in increased susceptibility to infection and allograft rejection. [106,107] The studies on the role of urobiome in post-kidney transplant recipients are limited. Rani *et al*. compared the urobiomes of kidney transplant recipients with that of healthy individuals. The urobiome was less diverse in kidney transplant recipients. *Escherichia coli* (*E.coli*), *Enterobacter* spp. were the predominant organisms isolated from such patients. On the other hand, in healthy controls, the urobiome was more diverse and non-pathogenic organisms such as *Propionibacterium, Mobiluncus, Corynebacterium* were detected. However, the above mentioned differences were not dependent on the pre-transplant kidney status.[108] In a prospective study on kidney transplant recipients by Fricke *et al*. it was found that the urobiomes were highly diverse during transplantation and persisted till six months post-transplant. It was also indicated that the urobiome composition were independent of kidney allograft function.[109] However, the clinical significance of urobiome variability in kidney transplant recipients remained unresolved. In a retrospective study on post-kidney transplant recipients with allograft dysfunction by Wu *et al*. it was found that *Corynebacterium* spp. was more prevalent.[106] However, the histopathological lesions in the allograft were not considered but the creatinine level was used as a marker for allograft dysfunction in the study. The association between urobiome composition and allograft biopsies in patients with interstitial fibrosis/tubular atrophy (IFTA) was done by Modena *et al*. In healthy females and pre-transplant female recipients, *Lactobacillus* spp. was predominant and in healthy males and pre-transplant male recipients, *Streptococcus* spp. was predominant.[110] Both the genera had a negative correlation with IFTA assessed in kidney allograft biopsies. *Achromobacter, Staphylococcus, Clostridiaceae, Anaeroglobus, Oligella, Dethiosulfovibrio, Massilia, Sneathia* were some of the organisms isolated from recipients prior to kidney transplantation.[21] (Table 3)

Table 3: Urobiomes in kidney transplant recipients (with IFTA)	
Clinical status of kidney transplant recipients	**Microorganisms**
1 month post-transplant	*Corynebacterium, Streptococcus, Lactobacillus, Prevotella* (Males)
	Gardnerella, Prevotella, Lactobacillus (Females)
6 months post-transplant	*Corynebacterium, Streptococcus, Lactobacillus, Prevotella (reduced)* (Males)
	Gardnerella, Prevotella, Lactobacillus (Females)
Kidney transplant recipients 12 months post-transplant	*Enterococcus, Streptococcus, Propionibacterium, Bacteroides, Lactobacillus, Escherichia, Salmonella, Proteus, Shigella, Ralstonia*

E.coli is the most common organism causing UTI in post-kidney transplant recipients and the predominance of pathogenic Gram-negative bacilli as a part of urobiome in such patients contributes to this issue. There are various studies[111,112,113] suggesting the negative influence of UTI towards kidney allograft function. Though the immunosuppression regimen implemented in these studies were calcineurin inhibitors, mycophenolate, steroids. None of the above-mentioned studies makes distinction in terms of pre-transplant kidney status or immunosuppressive regimen.

3. VIROMES OF THE URINARY TRACT

3.1. Urinary Virome in Health

Urine from healthy humans contained a variety of viruses.[114] It was found that most of them were phages in addition to human papillomaviruses (HPV) (>90% of subjects). [114,115] The research on virome is an emerging field. Though the methods employed for study have advanced, there are various challenges while working with metagenomic dark matter. It has already been found that many viruses are present in the renal and lower urinary structure.[116] Cohort research was conducted on 142 renal transplant recipients and an equivalent number of healthy individuals. Of the 37 viruses found, 29 were firstly detected in samples of human urine.[117] Thoroughly evolved viruses persist as medically undiagnosed, enabling a balance between the virus and the host posing benefit for both whereas pathogenic eukaryotic viruses increase their probability of transmission.[118] It is found that in around 30% of healthy individuals in African American population there is active replication of JC virus (JCV) in the urine associated with decreased rates of nephropathy.[116] Researchers focus on eukaryotic viruses such as HPV, JCV, BK virus, and Torque Teno virus (TTV).

3.2. Urinary Virome in Disease

Virome is found to be associated with UTIs. Most of them were due to bacteriophages and other eukaryotic viruses.[114] Viromes are gaining interest among transplant researchers as most of the viruses remain in a latent phase and undergo reactivation due to immunosuppression such as organ transplantation. However, the association of certain viromes with carcinogenesis is not completely understood.[119] One of the main factors contributing to organ rejection following renal transplantation is viruses. [120,121] The presence of JCV in urine protects against allograft rejection.[122] BK virus nephropathy (BKVN) is a serious complication after kidney transplantation. Rani *et al*. in a study on 22 kidney transplant recipients found the replication of the BKV virus in the urine followed by other polyomaviruses such as JCV and TTV. Viruses belonging to other families were also detected such as Adenoviridae, Anelloviridae, Papillomaviridae, and Herpesviridae. Viral polymorphisms linked to VP1, VP2 proteins, and large T antigens indicated varying pathogenicity of these viruses. Substantially, there were lower counts of BKV in the control group classified as serum BKV PCR-negative. About JCV and TTV, the differences between the recipient and control groups were statistically insignificant. In this study viruses such as Herpes simplex virus (HSV) type 1 and type 2 Epstein-Barr virus (EBV), and Cytomegalovirus (CMV), were not isolated from urine.[123] A study using metagenomic virome sequencing by Schreiber *et al*. showed that the JCV virus was present among donors' urine as well as recipients during kidney transplantation; a few weeks to months and/or a year after transplantation in beneficiaries. Donor-derived JCV strains were dominant as detected by a phylogenetic study.[124]

3.3. Bacteriophages in The Urobiome

Many studies on the bacteriophages in the gut microbiome suggest its contribution towards innate immunity against pathogenic bacteria and also stability of the bacterial community.[125,126,127] Bacteriophages are the predominant members of the human viromes.[128] However, there is currently no data to justify bacteriophage involvement in the urobiome.[129] According to a study by Brown-Jaque *et al*.[130] Forty six percent of urine samples had bacteriophages that attacked *E. coli* thereby shedding attention on the possibility of using bacteriophages to treat UTIs.[131]

4. ROLE OF FUNGI IN THE UROBIOME

Very little is known about mycobiome in the urine. Current technologies such as next-generation sequencing are not beneficial for analyzing the mycobiome.[11] There are studies that found interactions between the reagent used for mycobiome DNA extraction, amplification, and the non-fungal host DNA molecules. Hence, research is focused on the optimization of fungal extraction techniques from urine samples.[132] Other studies concluded the prevalence of *Candida* spp. in urine samples processed by conventional culture methods;[133,134] fungal organisms were detected in individuals with urological pelvic pain syndrome detected by biosensor system. Also, another finding of the study exposed an increasing prevalence of fungal disease from 3.9% in asymptomatic individuals to 15.7% in symptomatic patients.[135] Thus, further research is required in understanding the potential role of mycobiome and its role on health and disease.

5. METHODS FOR MICROBIOME ANALYSIS

The methods widely used for research and identification of microbiome are 16S rRNA gene sequencing, metagenomic sequencing and metabolomics; out of which 16S rRNA gene sequencing is the most commonly used method. The risk of horizontal gene transfer of 16s rRNA genes is less and 16S rRNA gene sequencing is cost-effective to analyse extensive microbiomes.[136] The disadvantage of this technique is its inability to differentiate between closely associated organisms.[137] Metagenomic sequencing provides comprehensive information on the entire genome of the microbes.[136] Metabolomics employing mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy for the study of metabolites produced by the microbiota helps us to understand the role of microbiomes in health and disease.[138]

6. CHALLENGES IN THE MICROBIOME STUDY AND UROBIOME EVALUATION

The basic approach to microbiome sampling is ideal samples that are non-invasive and have little or no cross-contamination. Urine is suitable for microbiome studies because of the simple collection methods, holds minimal debris and has few inhibitory substances that may affect amplification process, but traces of microbial contamination of the lower genitalia cannot be ruled out. On the other hand, urine is often high in salt, which helps keep organisms and nucleic acids viable for a while. Midstream urine is a convenient sample to recognise intra-bladder urobiome communications, such as those associated with bladder pain syndrome, interstitial cystitis, UTIs, bladder malignancy. This is in contrast to urine sample collection via suprapubic aspiration and transurethral catheterization. Despite being more invasive, these techniques are less likely to cause contamination with genital and rectal microbes.[20] Catheters and kidney stones often pass through the genitourinary tract, which can result in bacterial contamination of specimens. Surgical interventions such as nephrolithotomy for pre-operatively challenging renal calculi might help to provide precise and improved urobiome analysis. The initial site of clinical material removed is a crucial consideration if scientists want to investigate the relationship between bacterial makeup and stone illness. Although tidy, operating rooms are not completely sterile. Speaking with the surgeon about the substance's exact composition and removal techniques is also highly beneficial for avoiding contamination. Extracorporeal shock wave lithotripsy (ESWL) is a non-invasive method of retrieving smaller stone fragments. The urine following ESWL procedure could be hypothetically indicative of urobiomes from this site. Nevertheless, differentiating it from uropathogens causing urinary tract infections can be difficult. ESWL may potentially dislodge biofilms at the bladder, releasing bacteria hence, detailed understanding of the location of the stone sample is necessary.[139] EUCTs might serve as a tool for determining the microbiota's state but genomic studies have already been utilised to distinguish between gut, vaginal and urine microbiome.[140] A wider range of genes would be confirmed and gene libraries such as Kyoto Encyclopaedia of Genes and Genomes can verify their ontology (KEGG). This concept was put into practice in the work by Rani et al. stated above, where they discovered that among kidney transplant recipients who had regular STX/TMT prophylaxis, there were several particular genes associated with sulfamethoxazole/trimethoprim (SXT/TMT) resistance in the urobiomes.[108] In spite of numerous key discoveries on the

urobiome assembly in current years, it is still difficult to make broad inferences. Variations in the course of bladder cancer seem to be extremely obvious, probably having some prognostic consequences. However, the entire eukaryotic taxa that make up the urobiome (such as fungi and protozoa) have not yet been addressed. This is mostly because DNA extraction and additional analysis are difficult.

7. CONCLUSION

Though metagenomics has paved way for investigating microbiomes in sterile body sites, the most significant limitation is its inability to differentiate between live or dead organisms. The other drawbacks are the lack of standard sample collection procedures and DNA isolation protocols which greatly influence the outcome of the approach thereby making it hard to compare between various research centres. Hence, the urobiome is a ripe ground for upcoming research which will direct us towards profound improvements in the treatment of various urological disorders.

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9. CONFLICTS OF INTEREST

Conflict of interest declared none.

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