Urine flora imbalance and new biomarkers in prostate cancer and benign prostatic hyperplasia

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Abstract

Introduction: Microbial structure is closely associated with the initiation and development of various diseases. However, the roles of urine flora in prostate diseases, including prostate cancer (PCa) and benign prostatic hyperplasia (BPH), are still unclear.

Material and methods: In this study, clinical samples were collected from PCa (n = 21) and BPH (n = 19) patients and healthy people (n = 12). The analysis of urine flora DNA sequencing and hematological testing results between groups was performed using bioinformatic methods, including alpha and beta diversity analysis, and functional PICRUSt analysis.

Results: The results showed that the microbial structure in PCa and BPH differed from the healthy control. Abundance of *Escherichia coli* was higher in PCa and BPH patients, while probiotics, such as *Lactobacillus helveticus* and *Lactobacillus iners*, were lower. Moreover, beta diversity in the PCa group was significantly different from the control group, while alpha diversity was not. Spearman analysis showed that *Escherichia coli* was negatively correlated with *Lactobacillus helveticus* and *Lactobacillus iners*. Functional analysis showed that microbial imbalance was associated with energy metabolism in PCa, and with cell motility, energy metabolism, and intracellular trafficking, secretion, and vesicular transport in BPH. Moreover, microbial imbalance was associated with nervous disorders and infectious diseases in PCa, and with metabolic system, infectious diseases, and signal transduction in BPH.

Conclusions: Taken together, microbial imbalance may be associated with PCa and BPH. The increase of *Escherichia coli* was accompanied by the decrease of probiotics, such as *Lactobacillus helveticus* and *Lactobacillus iners*. These may be biomarkers for risk prediction and early treatment for prostate disease.

Key words: prostate cancer, benign prostatic hyperplasia, urine flora, microbial structure imbalance.

Introduction

Microbial imbalance is closely associated with the initiation and development of prostate diseases, such as prostate cancer (PCa) and benign

prostatic hyperplasia (BPH) [1, 2]. Imbalanced flora structures contribute to metabolic disruption in the urinary system and immune response, resulting in carcinogenesis [3]. Moreover, microbial structure imbalance promotes activation of the immune response and chronic inflammation and growth of epithelial and stromal tissues in the prostate, which are main features of BPH [4]. Increasing studies reveal that men with BPH are more at risk of PCa and PCa-related death [5–7]. However, whether this association indicates a causal link, shared risk factors or pathophysiological mechanisms, or detection bias, has not been fully elucidated. Urine flora is symbiotic microbial dwelling in human urinary system mucosa [8]. The changes of bacterial flora modulate chemoresistance for resistant pathogens, which is caused by biofilm formation, pressure from antibiotic treatment, and occasional contact with contaminated medical facilities [9, 10]. Thus investigating urine microbial structure may provide novel biomarkers for the diagnosis and early treatment of PCa and BPH.

Lactobacilli are probiotics inhabiting the human gastrointestinal or urogenital tract [11]. Lactobacilli play protective and probiotic roles in inhibiting the infection of the urinary system. Moreover, several studies have shown tumor-suppressing properties for certain *Lactobacillus* strains [12, 13]. Lactobacilli inhibit the progression of cancer via suppressing pathogens colonization, activating the immune response, directly promoting cancer cell death and antimutagenesis, adjusting carcinogen metabolism and protecting against oxidative-induced DNA degradation [14]. Interestingly, probiotic lactobacilli play a protective role in the urinary system and inhibiting the initiation and development of cancer [15]. *Lactobacillus rhamnosus* GG inhibits the tumor growth of bladder cancer [16]. *Lactobacillus* strains have natural killer activity of human peripheral blood mononuclear cells against prostate cancer cells [17]. However, the roles of *Lactobacillus iners* and *Lactobacillus helveticus* in PCa have not been elucidated.

In this study, we investigated the microbiota structure in PCa and BPH patients. The microbiota structure in PCa and BPH patients was differentiated from healthy control. *Lactobacillus iners* and *Lactobacillus helveticus* were negatively correlated with *Escherichia coli*. This may provide a new insight into the microbiota structure in PCa and BPH.

Material and methods

Sample collection

Clinical samples were collected from BPH or PCa patients (≤ 82 years old) diagnosed at the First Affiliated Hospital of Anhui Medical University from August 1st, 2017 to July 31th, 2018 (AMUYY[2017]0329). 12 healthy volunteers were recruited as healthy controls. The enrolled patients had been informed how to collect uncontaminated urinary samples. The enrolled patients were requested to provide 24-hour urine collections and freshly voided morning urine samples. The penis was washed with warm water and a 75%-alcohol tampon and collected using a 10-ml sterile tube without touching the interior wall. The enrolled patients had the first prostate biopsy or no biopsy for over 1 year. Patients receiving antibiotic therapies were excluded. The urine samples were kept in 10 ml sterile tubes and stored at –80°C. This study was approved by the Ethics Committee of the First Affiliated Hospital of Anhui Medical University. Each patient handed in informed consent.

DNA extraction

Total DNA was extracted with a QIAamp DNA Mini Kit (Qiagen, USA). DNA concentration was detected with an ultraviolet spectrophotometer (Thermo Electron Corporation, USA). The DNA samples were amplified in the V4 regions of the 16S rRNA.

DNA sequencing

PCR products were purified and recovered by a DNA Gel Recovery Kit in accordance with the previously validated protocol. The DNA samples were amplified with a 16S Metagenomic Sequencing Library Preparation Kit (Illumina, CA, USA). 16S rRNA V4 region was amplified in two step PCR protocols. PCR amplicon was performed with a gel extraction kit (Nippon Genetics, Japan). The sequences of the primer used were as follows: F: 5'-GTGCCAGCMGCCGCGG-3'; R: 5'-GGACTACHVGGGTWTCTAAT-3'. The library validation was conducted using the Illumina MiSeq system. Sequencing was conducted with HiSeq 2500 PE250 and analyzed with Sequence Analysis Viewer (Illumina).

Data analysis

Data were analyzed with CASAVA software (V1.8.2). Kruskal-Wallis, LEfSe and DEseq2 methods were applied to analyze the differences in abundance in samples. Urine bacteria taxonomy was assessed with individual operational taxonomic units (OTUs). Shannon's Index, Chao1, Observed Species, and PD Whole Tree index was applied to evaluate alpha diversity, and Bray-Curtis and Unweighted UniFrac for beta diversity.

Establishing prediction model

Partial least squares discriminant analysis (PLS-DA) was performed to distinguishes groups. For data closer to 1, PLS-DA analysis more reliable, and the abundance was more distinguishable.

Function prediction

Urine flora function prediction was performed using PICRUSt software with 16S species information and KEGG analysis, as previously described. According to PLS-DA analysis, urine floras of healthy control, BPH and PCa groups, and NOR groups were significantly differentiated into three independent clusters.

Statistical analysis

Data were analyzed with SPSS 19.0 and represented as mean ± SD. The difference among multiple groups was assessed by ANOVA. $P < 0.05$ was considered to be statistically significant.

Results

Demographic data

The age of the participants (n = 21) in the PCa group ranged from 53 to 78 (mean = 71.2). The mean age of the BPH group (n = 19, aged from 58 to 82) was 68.4. The mean age of the healthy group (n = 12, aged from 48 to 74) was 62.3. There was no significant difference in age.

DNA sequencing results

A total of 2,509,245 available rRNA reads were collected from clinical samples. The average reads were 48,255 ±3,762. After the data optimization, 1,546,248 unique sequences were obtained (Figure 1).

OTU analysis

After sequence clustering, a total number of 3586 OTUs were obtained, among which there were 1945 OTUs in PCa group, 1087 OTUs in BPH group, and 554 OTUs in healthy control group (Figure 1). Species abundance and evenness were evaluated by rank-abundance curves, which were applied to analyze the increase of the species and sample size. The smoothness of the curve reflected the uniformity of the species distribution. As shown in Figure 1, the smoothness of the rank-abundance curve predicted high evenness. Moderate species accumulation reflected that the species did not increase with increase in sample size.

Moreover, we determined the diversity of urine flora in the PCa group, PCa group, and healthy control group using OTUs, Shannon and Good's phy-

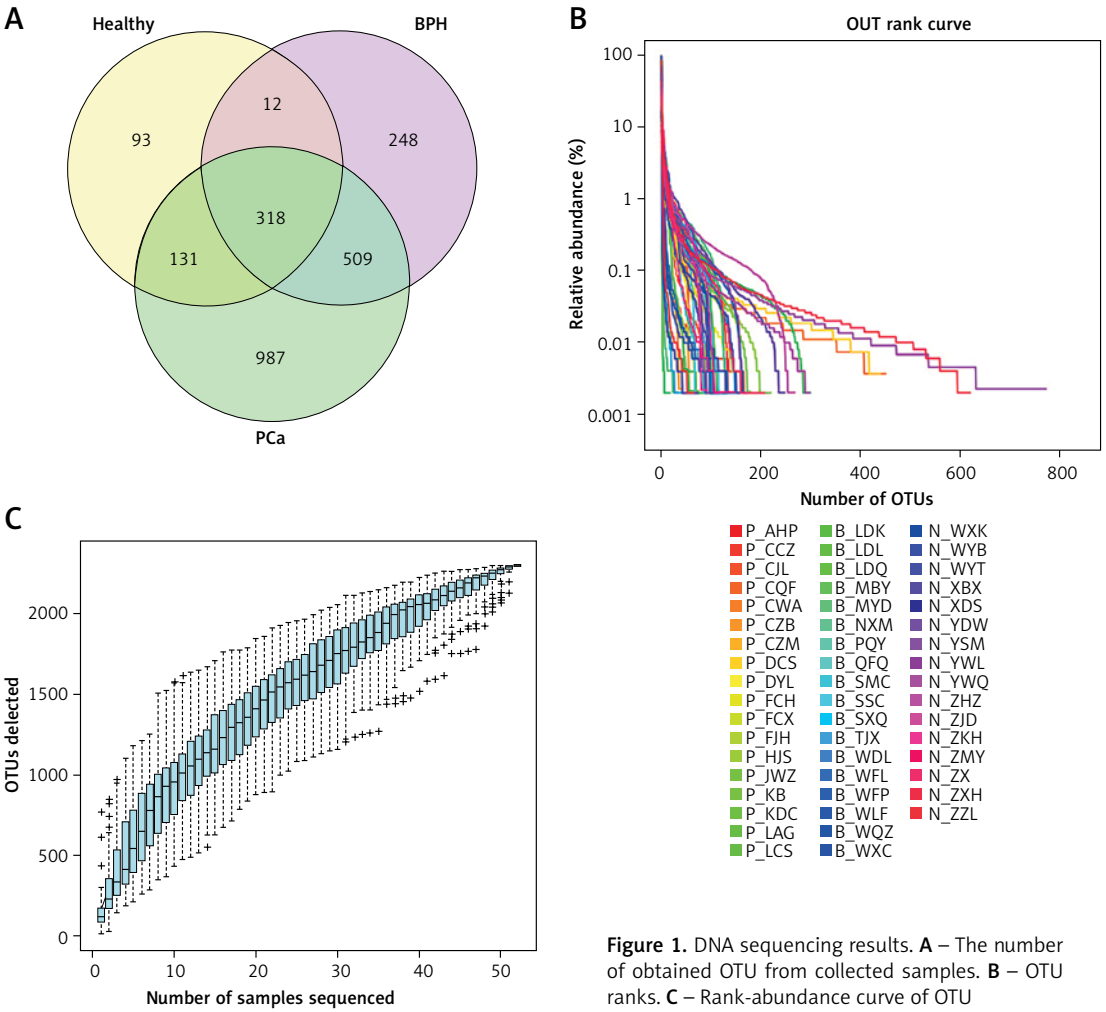

Figure 1. DNA sequencing results. **A** – The number of obtained OTU from collected samples. **B** – OTU ranks. **C** – Rank-abundance curve of OTU

logenetic diversity indices. As shown in Figure 2, the three groups showed no significant difference.

Microbial structure analysis

To investigate the microbial structure in urine of patients with PCa and BPH, the abundance of flora at phylum, genus, and species level was analyzed. As shown in Figure 3 A, the main phylum is *Crenarchaeota*, followed by *Tenericutes*, *Actinobacteria*, *Bacteroidetes*, and *Cyanobacteria*. In comparison with the healthy control group, the proportions of *Actinobacteria* in the PCa group and BPH were significantly lower. Additionally, the abundance of microorganisms in PCa and BPH groups was differentiated from the healthy control (Figures 3 B and C). It was found that the composition of the microorganisms in the three groups was distinctly different at different taxonomic levels. Moreover, in the top ten abundance at species level, abundance of *Streptococcus alactolyticus*, *Bacillus cereus*, and *Arthrobacter woluwensis* was lower in PCa in comparison with the healthy control, while that of *Atopobium vaginae* was higher. In BPH, *Streptococcus alactolyticus* and *Streptococcus alactolyticus* abundance was lower, but *Acinetobacter rhizosphaerae*, *Pseudomonas veronii*, and *Brevundimonas vascularis* abundance was higher. Moreover, *Lactobacillus iners* and *Lactobacillus helveticus* levels were significantly lower in PCa and BPH patients, while *Escherichia coli* abundance was higher (Figures 4 A and B).

The results from PLS-DA analysis showed that the urine floras in the PCa group, BPH group and healthy control group were differentiated, and that the prediction model was successfully constructed. Abnormal levels of urine flora in PCa and BPH were

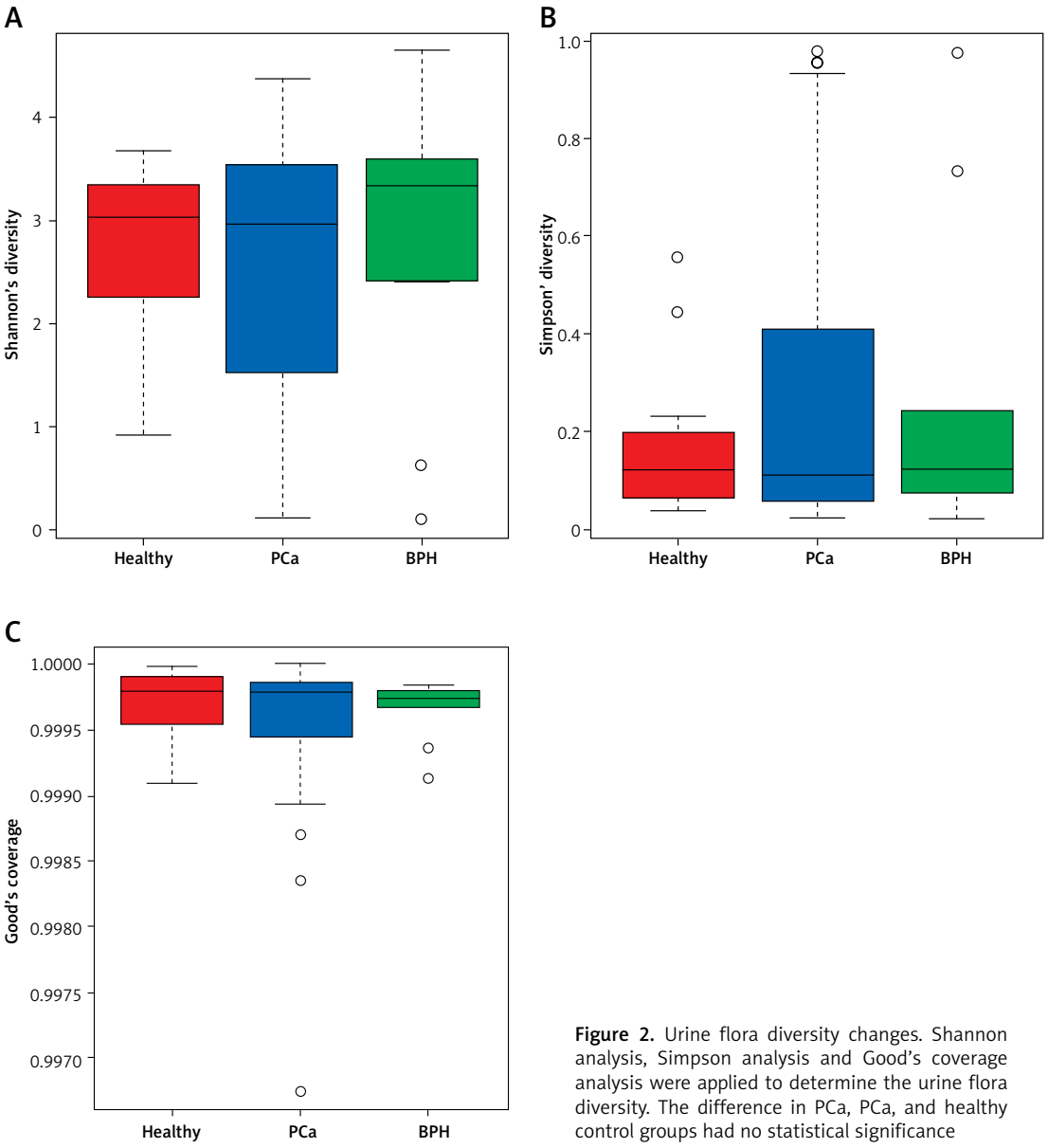

Figure 2. Urine flora diversity changes. Shannon analysis, Simpson analysis and Good's coverage analysis were applied to determine the urine flora diversity. The difference in PCa, PCa, and healthy control groups had no statistical significance

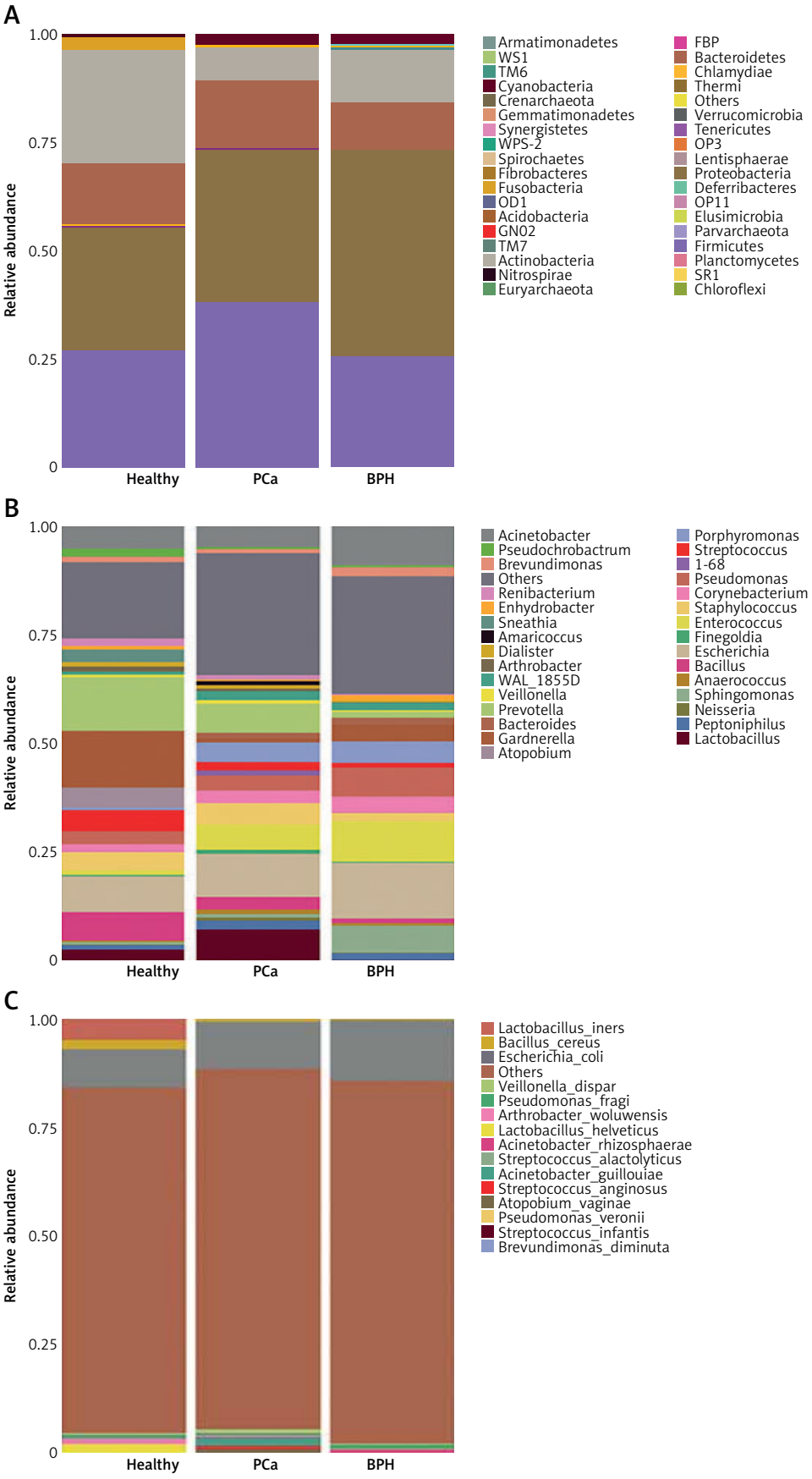

Figure 3. Relative taxa abundance in PCa and BPH. **A** – Relative taxa abundance at the phylum level. **B** – Relative taxa abundance at the genus level. **C** – Relative taxa abundance at the species level

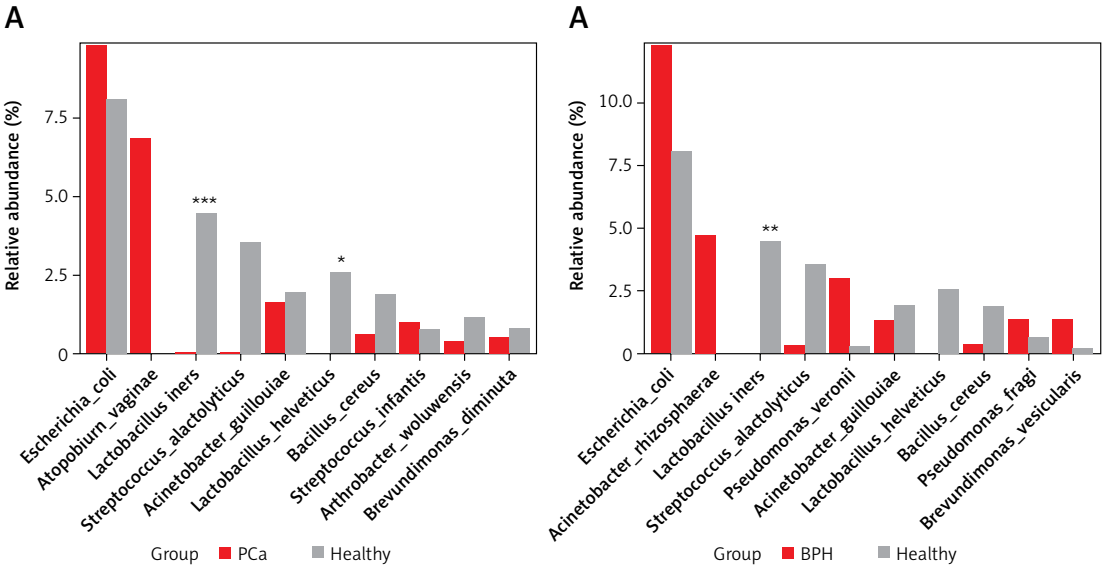

Figure 4. Top ten taxa at species level. **A** – Comparison of taxa abundance between healthy control and PCa patients. **B** – Comparison of taxa abundance between healthy control and BPH patients. *$P < 0.05$, **$p < 0.01$ vs. Healthy control group

strong prediction factors and may be risk factors for prostate diseases (Figure 5). For instance, *Escherichia coli* level was increased in both BPH and PCa, which suggested that an aberrantly high level of *Escherichia coli* may be conducive to BPH and PCa.

Urine flora correlation analysis

A correlation heat map of healthy control, BPH, and PCa between intestinal flora at the species level was constructed. As shown in Figure 6, *Escherichia coli* was negatively correlated with *Lactobacillus helveticus* and *Lactobacillus iners*, and positively correlated with *Streptococcus infantis*, *Acinetobacter guillouiae* and *Pseudomonas fragi*.

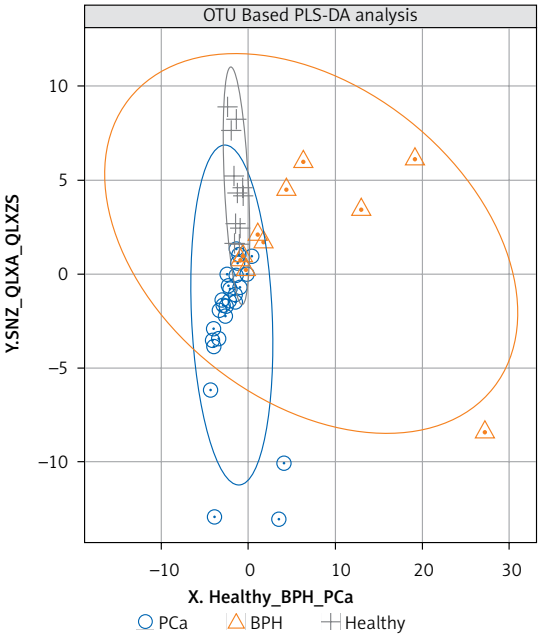

Figure 5. PLS-DA analysis of the microbiome among healthy control, PCa and BPH patients. The urine floras in PCa group, BPH group and health control group were independently differentiated. The different patterns, or colors, varied among groups

Urine microbial function prediction

To investigate the microbial structure in PCa and BPH patients, PICRUSt, 16S, PCA analysis, KEGG-pathway, and Pearson analysis were performed to detect the potential of microbial imbalance in function changes. PICRUSt and PCA analysis applied to predict functions (Figure 7). At the first KEGG pathway level, the urine microbial function of BPH was associated with human diseases and cellular processes, and the urine microbial function of the healthy control group was associated with the original system. At the second KEGG-pathway level, the urine flora function of PCa was associated with nervous disorders and infectious diseases; the urine flora in BPH patients was associated with signal transduction, infectious disease, and metabolism, while the urine microbial function of healthy control was mainly associated with the immune response, signaling interaction, and endocrine system. These results suggested that PCa and BPH patients are at greater risk of nervous disorders and infectious diseases. Moreover, the urine flora function of PCa was associated with energy production and conversion, which is one of the hallmarks of cancer. The urine flora in BPH patients was associated with cell motility, energy production and conversion, and intracellular trafficking, secretion, and vesicular transport, which suggested that flora structure may be a potential biomarker for PCa and BPH.

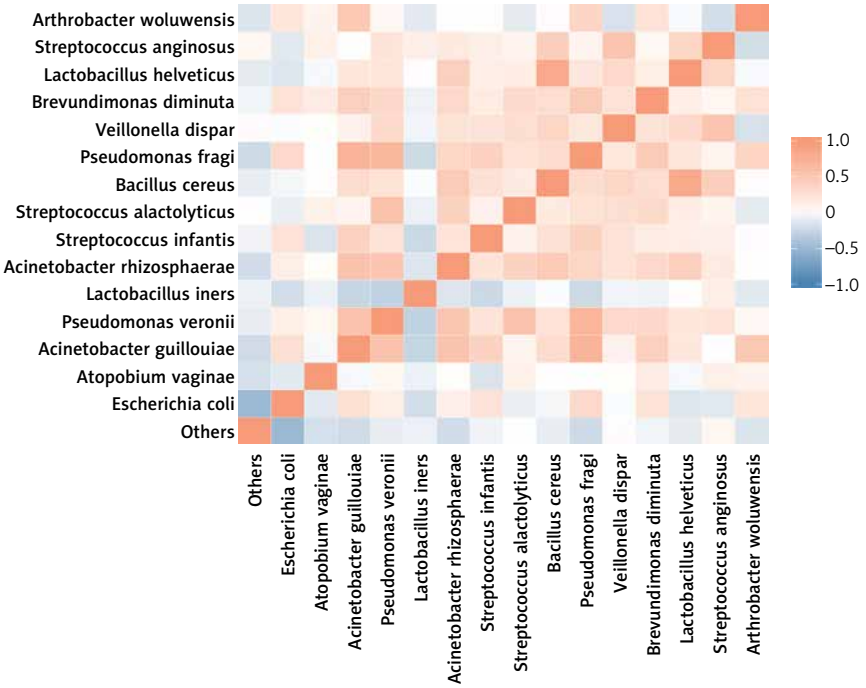

Figure 6. Urine flora correlation analysis. The urine flora correlation analyzed by a correlation heat map method. *Escherichia coli* was negatively correlated with *Lactobacillus helveticus* and *Lactobacillus iners*, and positively correlated with *Streptococcus infantis*, *Acinetobacter guillouiae*, *Pseudomonas fragi*

Discussion

Increasing evidence reveals that microbiota structures are associated with various urethral diseases, including PCa and BPH [1, 2]. Flora imbalance contributes to the progression of BPH and PCa. Thus to investigate the urinal flora structures in PCa and BPH was of vital importance. In this study, urinal flora structures in PCa and BPH were differentiated with the healthy control. Additionally, the biological function was investigated, which may provide a potential biomarker for PCa and BPH.

In this study, urinal flora structures varied with healthy control, PCa and BPH patients. At the phylum level, Proteobacteria level was significantly increased in PCa and BPH, while *Actinobacteria* level was decreased. The aberrant increase of Proteobacteria is associated with the initiation and progression of BPH and PCa. Interestingly, Proteobacteria extensively participates in urinary tract infection [18]. Lipopolysaccharide in opportunistic Proteobacteria contributes to tumorigenesis via secreting pro-inflammatory factors though activating the host's pattern-recognition receptors [19, 20]. Thus opportunistic Proteobacteria induced systemic inflammation is conducive to disease onset or progression [21]. *Actinobacteria* produces new secondary metabolites with pharmaceutical applications [22]. The natural products secreted from *Actinobacteria* may have potential for future drugs against crucial diseases such as cancer [23]. For instance, *Anthracyclines* produced by *Actinobacteria* is effective in cancer therapy [24]. Thus the decrease of *Actinobacteria* in urinal microbiota structure may be associated with the progression of BPH and PCa.

Escherichia coli and opportunistic pathogens, such as *Acinetobacter rhizosphaerae*, *Pseudomonas veronii*, *Brevundimonas vecicularis*, were increased in urine microbiota of BPH or PCa patients. However, the abundance of anti-inflammation bacteria *Lactobacillus helveticus* and *Lactobacillus iners* was decreased in BPH and PCa patients.

Escherichia coli was increased in BPH and PCa patients. *Escherichia coli* plays a crucial role in energy production and conversion [25]. The *Escherichia coli* metabolic network copes with changing proteomic demands of energy biogenesis and biomass synthesis under different growth conditions. Therefore, the abnormal cell growth in BPH and PCa may facilitate the abundance of *Escherichia coli* [26]. Interestingly, the host cell inflammatory responses, in turn, promote *Escherichia coli* resilience. Moreover, *Escherichia coli* induces persistent colonization, exacerbates inflammation and triggers carcinogenesis [27]. *Escherichia coli* is a crucial factor for the development of colorectal cancer, breast cancer, gynecological cancer, and prostate cancer [28–31]. The enrichment in *Escherichia coli* contributes to host cell inflammatory responses and intestinal dysbiosis, promoting the progression of inflammatory bowel disease (IBD) and CRC [28]. Uropathogenic

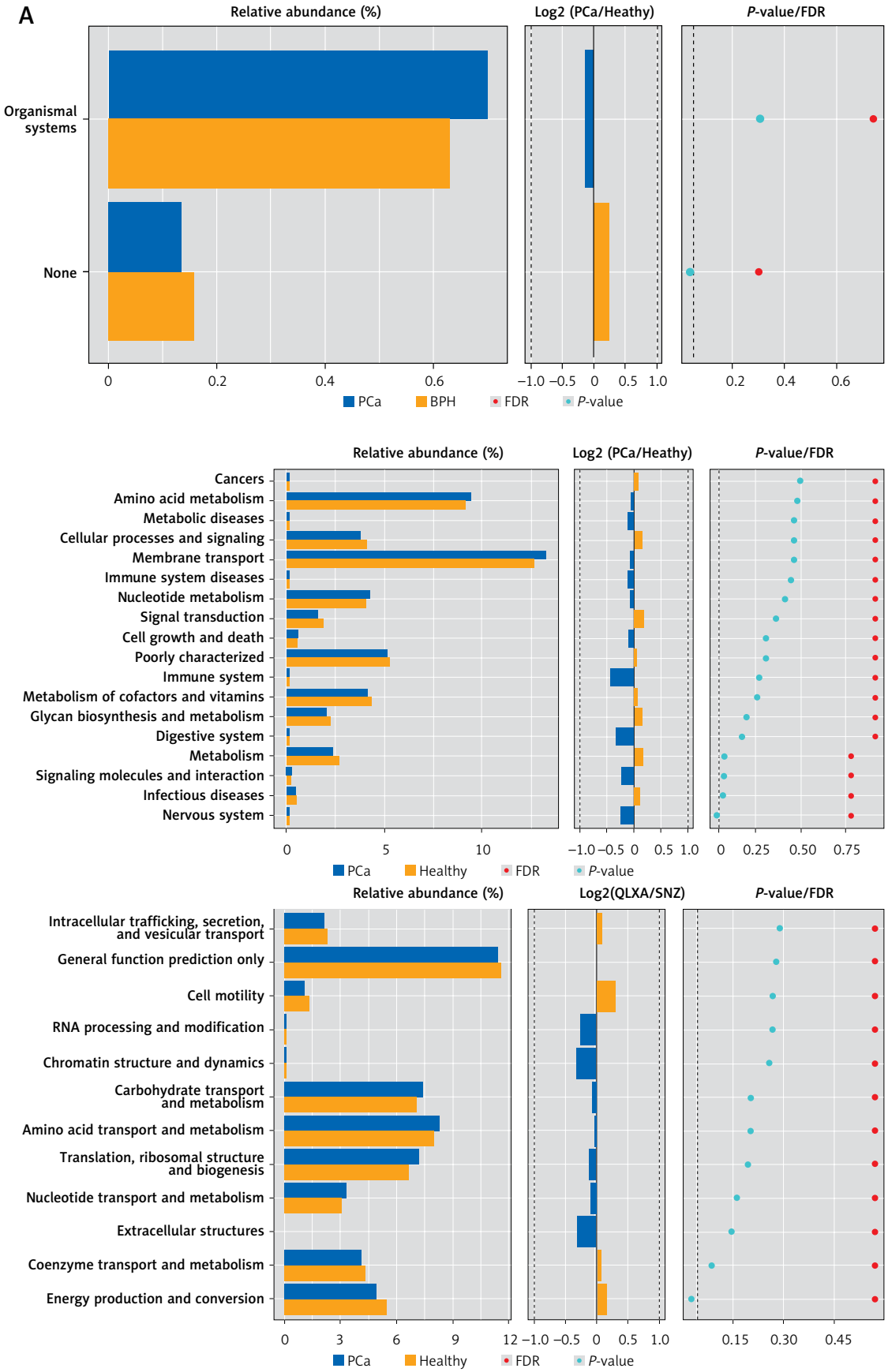

Figure 7. PICRUSt analysis among healthy control, PCa and BPH patients. **A** – KEGG-pathway analysis at first pathway level. The urine microbial function of BPH was associated with human diseases and cellular processes, and the urine microbial function of the healthy control group was associated with the original system

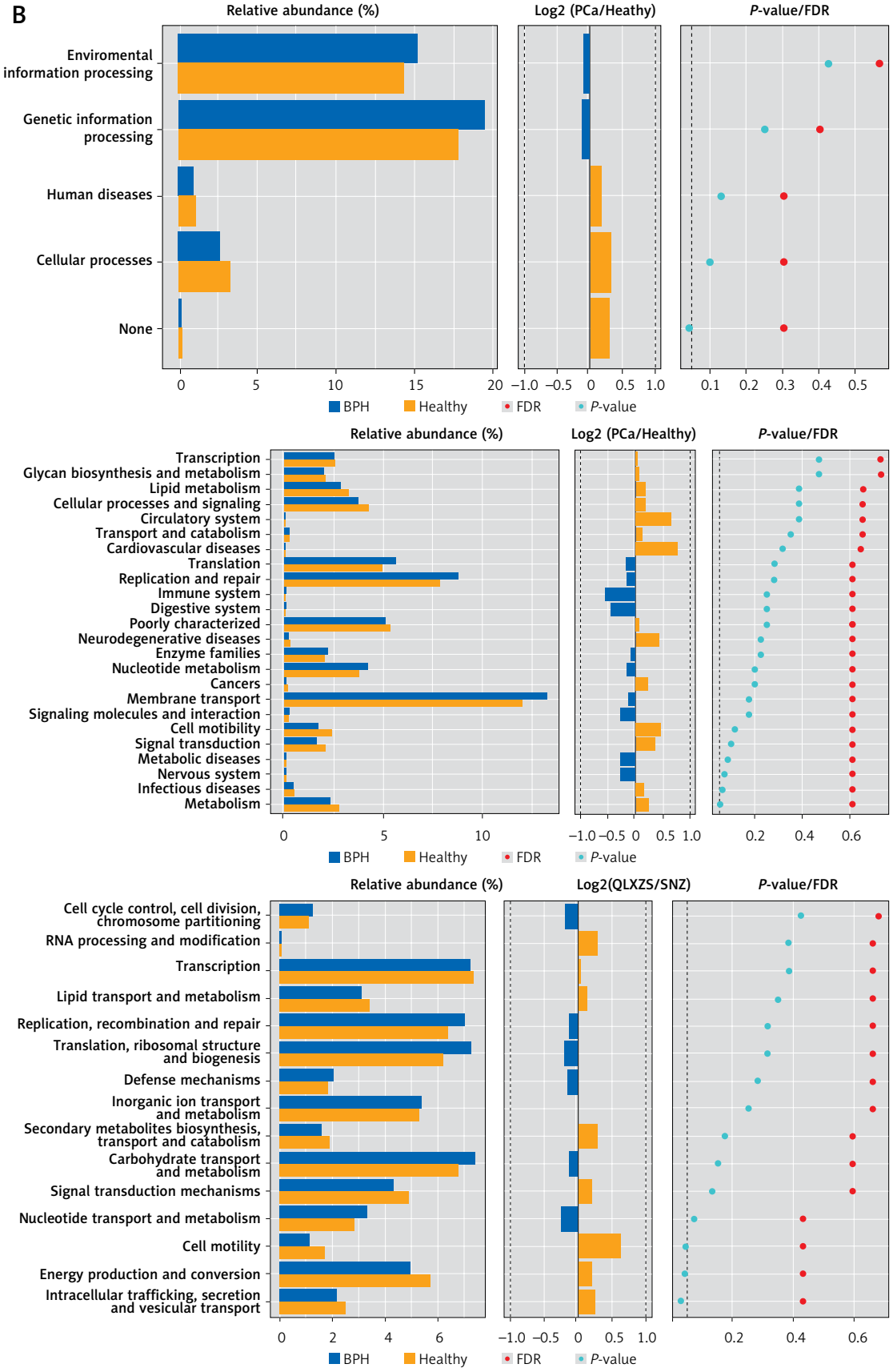

Figure 7. Cont. **B** – KEGG-pathway analysis at second pathway level. The urine flora function of PCa was associated with nervous disorders and infectious diseases; the urine flora in BPH patients was associated with signal transduction, infectious disease, and metabolism. The urine microbial function of healthy control was mainly associated with immune response, signaling interaction, and endocrine system. The urine flora imbalance was associated energy production and conversion in PCa and cell motility, energy production and conversion, and intracellular trafficking, secretion, and vesicular transport in BPH

Escherichia coli invasion is conducive to bacterial prostatitis and the deterioration of PCa [32]. Interestingly, the cancer risk induced by *Escherichia coli* is inflammation-dependent. Specific environmental conditions (e.g. inflammation), in spite of the enrichment in *Escherichia coli*, are necessary for carcinogenesis in CRC. This may form a feedback loop for carcinogenesis. Then the inhibition of *Escherichia coli* suppresses host cell inflammatory responses and abates the oncogenic effects of *Escherichia coli*. Previous studies revealed that lactobacillus probiotics offer an antibiotic-sparing prevention strategy for urinary tract infections via inhibiting *Escherichia coli* [33]. However, the roles of *Lactobacillus* in the prostate have not been fully elucidated.

Lactobacillus iners plays protective roles in infectious disease and cancer [34]. Lower abundance of health-supporting lactobacilli, especially *Lactobacillus iners*, is associated with progression of prostatitis [35]. In this study, *Lactobacillus iners* was decreased in BPH and PCa patients. Therefore, lack of *Lactobacillus iners* may be associated with the development of PCa. *Lactobacillus helveticus*, a crucial member of *lactobacillus* probiotics, possesses anti-cancer and antioxidant properties [36]. It promotes the survival of gastrointestinal transit, adhesion to epithelial cells, and pathogen-antagonization. Moreover, *Lactobacillus helveticus* suppresses progression of infectious diseases and attenuates host immune responses and microbiota structures [37]. In this study, the abundance of *Lactobacillus helveticus* was decreased in PCa. Additionally, *Lactobacillus helveticus* metabolites exert anti-proliferative function. A low level of *Lactobacillus helveticus* may be conducive to the development of BPH and PCa. *Lactobacillus helveticus* NS8 suppresses the carcinogenesis and degree of hyperplasia, due to its anti-proliferation and anti-inflammation outcomes before tumor formation [38]. *Lactobacillus helveticus* alleviates organisms' dysbiosis by inducing beneficial commensal microbes and inhibiting oncomicrobes. In this study, the abundance of *Lactobacillus helveticus* and *Lactobacillus inners* was negatively correlated with *Escherichia coli*. Hence, *Lactobacillus helveticus* and *Lactobacillus iners* may be a novel strategy for restoring prostate function and inhibiting *Escherichia coli*. Moreover, Eslami *et al*. evidenced that probiotics prevent proliferation and metastasis of colorectal cancer and improve the effectiveness and safety of cancer therapy [39]. Further study will investigate the potential of *Lactobacillus helveticus* or *Lactobacillus iners* PCa therapy.

However, there are several limitations of this study. First, the number of participants in this study was very small. Possible tissue samples are needed to make the results more convincing. Further studies will recruit more volunteers. Urine samples for DNA testing obtained through a percutaneous approach will make the results more accurate. *In vivo* and *in vitro* studies will be performed to investigate the association between bacterial community prostate diseases.

In conclusion, the increase of pro-inflammation *Escherichia coli* and the reduction of anti-inflammation and anti-proliferation *Lactobacillus helveticus* and *Lactobacillus inners* were the main features of BPH and PCa. Ecological dysbiosis of the bacterial community may be associated with the inflammatory response and abnormal proliferation. Therefore, further investigation of the potential roles of health-supporting lactobacilli in ecological dysbiosis, the inflammatory response, and abnormal proliferation in prostate may provide an individualized strategy for PCa.

Acknowledments

Shuiping Yin and Dandan Xu contributed equally in this work.

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Conflict of interest

The authors declare no conflict of interest.

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