Gut microbiota bridges the inadequate consumption of fruits and bladder cancer

Canxia He1, Baolong Li2, Lei Huang1, Xiaodong Liu1, Peijun Tian1, Yongping Bao3, Minghua Ren4 and Yujuan Shan1

1Department of Food Science and Engineering, School of Chemistry and Chemical Engineering, Harbin Institute of Technology, Harbin 150090, China
2Center of Safety and Evaluation of Drugs, Heilongjiang University of Chinese Medicine, Harbin 150030, China
3Norwich Medical School, University of East Anglia, Norwich, NR4 7UQ, UK
4Department of Urinary Surgery, the First Affiliated Hospital of Harbin Medical University, Harbin 150001, China

Correspondence to: Yujuan Shan, email: yujuan72@163.com
Baolong Li, email: lbl73@163.com
Minghua Ren, email: renminghua1972@163.com

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ABSTRACT

Intake of fruits and vegetables has been negatively correlated with bladder cancer (BC), while its underlining mechanism remains unknown. Here, the numbers of Clostridium cluster XI and Prevotella were diminished in BC patients whose fruits intake were largely lower than that in healthy subjects. Quantities of butyric acid in feces and iso-butyric acid in cecal contents, were reduced in BC patients and BC mice, respectively. Furthermore, the loss of goblet cells and visible breakage of the mucosal epithelium in the colon and cecum as well as the downregulations of zo-1/occludin/claudin-1 were obviously observed. The concentrations of lipopolysaccharide and D-lactic acid, two sensitive markers of gut permeability, were also increased. The concentrations of interleukin-6, interleukin-22 and secretory immunoglobulin A, key players in gut immunity, were significantly elevated in BBN-induced BC mice. In conclusion, there are definitely existed an imbalance of the gut microbiota in BC which possibly bridged the inadequate consumption of fruits and carcinogenesis of BC.

INTRODUCTION

Urothelial bladder cancer (BC) generally originates from urothelium, and transitional-cell carcinoma which is accounting for over 90% of bladder cancer represents the most frequent histological type [1, 2]. Approximately 75% of the newly diagnosed patients are considered as non-muscle-invasive bladder cancer belonging to T1 or T2 of Tumor, Node, Metastasis (TNM) stage. And 25% of the patients are muscle-invasive or metastatic diseases belonging to T3-T4 of TNM stage with bad prognosis [3]. Besides the cigarette smoking and occupational exposures to carcinogen [4], dietary factors, such as low consumption of fruits and vegetables as well as high consumption of the processed meat positively associated with the BC risk [1, 5, 6], whereas its underlined mechanisms remain unknown.

Very recently, some studies began to take considering about the relationship between urinary bacterial communities and urinary tract. A small pilot study revealed an enrichment of Streptococcus in urine samples from BC patients \( (n = 8) \) [7]. One possible mechanism is that microbiota produces proteases and then contacts with epithelium. These enzymes function as extracellular virulence factors in the host tissue degradation, evasion and destruction of host physical barriers. The bacterial invasion of tissues induces inflammation, generation of oxygen radicals that driven cancer and cancer recurrence [8].

Almost 99% of the microbial mass is located within the intestinal tract, and the microbiota and its host have co-evolved into a complex ‘super-organism’ [9–11]. Moreover, gut microbiota exerts not only local effects but also long-distance effects on the host. To communicate
with distant organs, gut microbial signals should be firstly transmitted across the intestinal epithelium [12]. Failure of the intestinal barrier will cause the leakage of undesirable solutes, pathogenic microorganisms and toxins, which subsequently lead to inflammation and immune activation [13]. Inflammation is an important driving force that shapes the microbial composition-for example, expansion of Enterobacteriaceae has been reported in patients with chronic inflammation [14]. Specifically, there is a feed-forward relationship among inflammation, barrier failure, the gut microbiota and carcinogenesis.

The human colonic microbiota produces an enormous quantity of molecules that impact on the gut homeostasis [15]. Diet qualitatively and quantitatively influences the gut microbial communities, and in consequence, the microbial-derived molecules presented in gut lumen [16]. Dietary carbohydrates that are not digested by the host are fermented in the colon into short-chain fatty acids (SCFAs) such as acetate, propionate and butyrate [17]. Most SCFAs are locally produced in large intestine by specific bacterial species. For instance, butyrate formation occurs in certain Firmicutes bacteria, and acetate is produced by most anaerobes. Approximately 80-90% of SCFAs are absorbed from the colonic lumen and partly utilized by colonic epithelial cells, others will be transferred to the circulation or excreted in feces [18]. Therefore, protective effects by SCFAs are not only present in intestinal mucosa but also extended to other compartments of the body. SCFAs have been shown to be associated with reduced risks of diseases such as inflammatory bowel disease and cancer for its antineoplastic, anti-inflammatory and immunomodulatory properties [19, 20]. Moreover, butyric acid presents the direct anticancer effect on bladder cancer cell by inhibiting cell growth and inducing apoptosis in vitro [21, 22].

Although epidemiological evidence implied the intake of fruits and vegetables was negatively associated with bladder cancer, the underlined mechanism remains unclear. We hypothesized that low consumption of fruits and vegetables may influence the composition of intestinal flora which, in consequence, initiated the carcinogenesis of the urothelial bladder epithelium. We aim to profile the characteristics of gut microbiota in bladder cancer, which will be helpful to uncover the diet-associated mechanism of bladder cancer.

RESULTS

Demographics and lifestyle-related factor analysis

Diet has an influence on the gut microbiota’s composition [23]. Moreover, diverse studies have demonstrated that cigarette smoking and dietary factors such as the decreased intakes of fruits and vegetables were associated with an increased risk of BC [24]. Thus, we evaluated health-related issues including food intake (vegetables, grains and fruits), frequency of weekly vigorous exercise, smoking and drinking status for all individuals. All of the characteristics and lifestyle factors for BC patients (n = 26, 18 males and 8 females) and healthy controls (HCs) (n = 16, 7 males and 9 females) were shown in Table 1. No significant differences were observed in the aspect of age, sex, body mass index and percent of overweight individuals between two groups. Also, the ratios (scores from 0 to 4) of smoking and drinking in two groups showed no significance (p = 0.960 for smoking, p = 0.206 for drinking). Data from food questionnaires indicated that the fruit intake in the BC group was markedly reduced in contrast with that in the HC group (the ratio for fruit scores from 0 to 3, 14:8:4:0 for the BC group versus 2:9:5:0 for the HC group, p = 0.026). (Supplementary Table 2)

Changes in fecal microbial communities and microbial components in BC patients and BBN-induced BC mice

Firstly, 83815 sequences were obtained for high throughput sequencing. Then, a total of 707, 02 optimized sequences were used for downstream analysis (on average, 14140 ± 1855 reads per sample) and were clustered into 3,233 operational taxonomic units (OTUs). The results of richness diversities, Shannon index, and Simpson rarefaction plot indicated that bacterial diversities in the BC groups were reduced (Supplementary Figure 1). A summary of the species richness in all groups was shown in Supplementary Table 1.

Due to the decreased bacterial diversities in BC patients, the detailed distributions of fecal microbial communities at the phylum level were studied (Figure 1A). The main differences between the HC and BC groups were attributed to Bacteroidetes and Firmicutes, which totally accounted for up to 85% of the overall on average. The abundance of bacteria at the class level in all groups were presented in Figure 1B. The results of UniFrac principal coordinate analysis (PCoA) (Figure 1C) indicated that the fecal microbial communities of BC patients and HCs were obviously separated from P_1, P_2 and P_3 (43.3%, 33.5% and 17.4% of the explained variance, respectively).

Twelve dominant bacterial groups, mainly belonging to the phyla Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria, were analyzed by real-time qPCR (Table 2). The numbers of domain Bacteria, Clostridium cluster XI and Prevotella in BC patients were significantly lower than those in the HC group (p < 0.05). Other microorganism such as Clostridium cluster I and Faecalibacterium prausnitzii showed no significance between the two groups.

To further confirm the results in BC patients, BBN was used to chemically induce the bladder carcinogenesis. Bladder index and liver index were significantly increased
Table 1: Demographic characteristics between bladder cancer cases and healthy people

| Characteristic                  | HC (N = 16) | BC (N = 26) | p value |
|--------------------------------|-------------|-------------|---------|
| Ages(years)/range\(^1\)        | 58.46 ± 10.56 | 62.19 ± 9.79 | 0.162   |
| Sex [% (n)]                    |             |             | 0.095   |
| Male                           | 43.75 (7)   | 69.23 (18)  |         |
| Female                         | 56.25 (9)   | 30.77 (8)   |         |
| BMI (kg/m\(^2\))\(^1\)         | 23.75 ± 3.07| 24.29 ± 3.30| 0.606   |
| Overweight [% (n)]\(^2\)       | 37.50 (6)   | 34.62 (9)   | 0.852   |
| Smoking [% (n)]\(^2,3\)        |             |             | 0.960   |
| 0                              | 6.25 (1)    | 7.69 (2)    |         |
| 1                              | 18.75 (3)   | 26.92 (7)   |         |
| 2                              | 0.00 (0)    | 3.85 (1)    |         |
| 3                              | 6.25(1)     | 3.85 (1)    |         |
| 4                              | 68.75 (11)  | 57.69 (15)  |         |
| Alcohol [% (n)]\(^2,3\)        |             |             | 0.206   |
| 0                              | 6.25 (1)    | 15.38 (4)   |         |
| 1                              | 43.75 (7)   | 15.38 (4)   |         |
| 2                              | 0.00 (0)    | 3.85 (1)    |         |
| 3                              | 0.00 (0)    | 0.00 (0)    |         |
| 4                              | 50.00 (8)   | 65.38 (17)  |         |
| Weekly vigorous exercise [%(n)]\(^2,3\) | 93.75 (15) | 76.92 (20) | 0.163   |
| 1                              | 62.5 (1)    | 3.85 (1)    |         |
| 2                              | 0.00 (0)    | 19.23 (5)   |         |
| Vegetables [% (n)]\(^2,3\)     |             |             | 0.381   |
| 0                              | 0.00 (0)    | 0.00 (0)    |         |
| 1                              | 93.75 (15)  | 100.00 (26) |         |
| 2                              | 6.25 (1)    | 0.00 (0)    |         |
| 3                              | 0.00 (0)    | 0.00 (0)    |         |
| Grains [% (n)]\(^2,3\)         |             |             | 0.106   |
| 0                              | 0.00 (0)    | 0.00 (0)    |         |
| 1                              | 68.75(11)   | 50.00 (13)  |         |
| 2                              | 25.00 (4)   | 50.00 (13)  |         |
| 3                              | 6.25 (1)    | 0.00 (0)    |         |
| Fruits [% (n)]\(^2,3\)         |             |             | 0.026   |
| 0                              | 12.50 (2)   | 53.85 (14)  |         |
| 1                              | 56.25 (9)   | 30.77 (8)   |         |
| 2                              | 31.25 (5)   | 15.38 (4)   |         |
| 3                              | 0.00 (0)    | 0.00 (0)    |         |
| Tumor stage                    |             |             |         |
| Ta                             | 4           |             |         |
| T1                             | 12          |             |         |
| T2                             | 7           |             |         |
| T3                             | 3           |             |         |

\(^1\)Data were expressed as the mean ± SD.\(^2\)Categorical variables were compared by a chi-square test or Fisher’s exact test.\(^3\)Scores for lifestyle information were according to Chen et al. [49].
in BC mice compared to HC mice (Figure 2A) (The hematoxylin and eosin staining slides for the liver were shown in Supplementary Figure 2). According to the histological evaluation by the professional pathologist, 70% (7 in 10) of mice with bladder cancer belong to noninvasive cancer (pTa), 20% (2 in 10) were of the invaded the lamina propria (pT1), and 10% (1 in 10) were of the invaded the muscularis propria (pT2) (Figure 2B). Clustering and principal component analysis (PCA) were performed based on the polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) data. The results of PCA revealed that the structure of the gut microbiota was significantly altered in BC mice (Figure 2C).

Decreased concentrations of SCFAs in BC patients and BBN-induced BC mice

The microbiota is enriched in genes relevant for dietary nutrient absorption [25]. To further clarify the possible and potential links between the decreased quantities of Clostridium cluster XI & Prevotella and the lower fruit consumptions in BC patients, we detected the quantities of SCFAs, the fermented metabolites of undigestible carbohydrates in the colon and cecum. As shown in Figure 3A, the contents of butyric acid in the BC group (69.38 ± 45.11 μmol/g) were lower than those in the HC group (125.39 ± 39.74 μmol/g) (p < 0.05). To further discover if the lower fruit intake is consequently associated with the decreased levels of butyrate, the correlation analysis was performed in the patients and healthy controls. According to the r value, butyric acid concentration is positively and significantly correlated with fruits consumption (Figure 3B). In BBN-induced BC mice (Figure 3C), the concentrations of SCFAs, especially iso-butyric acid and valeric acid in the BC mice were much lower than those in controls (0.90 ± 0.50 μmol/g versus 0.42 ± 0.16μmol/g for iso-butyric acid, 0.63 ± 0.15 μmol/g versus 0.28 ± 0.26 μmol/g for valeric acid, both p < 0.05).

Figure 1: The composition of the fecal microbiota in the BC patients and healthy controls. (A) The compositional gut microbiota profiles were showed at the phylum level in the healthy control group and four subgroups of BC patients (each color represents one bacterial phylum). (B) The heatmap of specimens showed the relative abundance of main bacteria at the class level. (C) PCoA-estimated gut microbiota in the healthy control group and four subgroups of BC patients.
Excessive lipopolysaccharide and D-lactic acid in bladder cancer due to the deficiency of the gut barrier

SCFAs, especially butyrate, act as a key energy source for intestinal epithelial cells and impact on mucosal integrity by stimulating the growth of intestinal epithelial cells [26]. LPS and D-lactic acid, known as two sensitive markers of gut permeability, could penetrate across the intestinal epithelium when the intestinal barrier is impaired [27–29]. As shown in Figure 4A, the concentrations of LPS and D-lactic acid in the BC group were both significantly higher in contrast with that in the HC group (7.89 ± 2.85 ng/mL versus 3.19 ± 1.00 ng/mL for LPS, 49.29 ± 46.95 μmol/mL versus 14.45 ± 4.67 μmol/mL for D-lactic acid, both \( p < 0.05 \)).

High levels of LPS and D-lactic acid were detected in BC mice (Figure 4B). At the same time, diverse damages in the intestinal epithelium, such as the loss of goblet cells and crypts, breakage of the surface epithelium and inflammatory lesions, were observed in BC mice (Figure 4C; b&d for colon, f&h for cecum). Histological defects (in the colon and cecum) were analyzed based on the histological score, (Supplementary Table 4) and the scores were nearly 6-fold higher in the BC group (1.33 ± 0.33 versus 13.33 ± 0.33 for colon, 2.33 ± 0.88 versus 12.67 ± 0.67 for cecum, both \( p < 0.05 \)). Zo-1, claudin-1 and occludin, the structural basis of intestinal barrier, were all decreased in the colon and cecum from BC mice at transcriptional levels (Figure 4D).

Up-regulation of cytokines and secretory immunoglobulin A (SIgA) in BBN-induced BC mice

To evaluate the effect of deficient intestinal barrier integrity on the innate immune and inflammatory responses in the host, nine related interleukins were measured in mice. These inflammatory factors are classically used for assessing the differentiation of naïve T cells into the Th1, Th22 and Th17 types of immune responses [30]. Among the 9 interleukins, the concentration of interleukin-6 (IL-6) was dramatically increased by nearly 6-fold (5.96 ± 1.98 pg/mL versus 30.51 ± 9.45 pg/mL) and almost 2-fold of IL-22 in BC mice (Figure 4E, both \( p < 0.05 \)). Other cytokines (IFN-γ, IL-1β, IL-10, IL-23, IL-12p70, IL-17A, TNF-α) were not significantly altered in BC mice (Supplementary Figure 3). The concentration of SIgA, the predominant immunoglobulin in mucosal compartments [31], was elevated by nearly 2-fold in the BC mice compared to the control mice (\( p < 0.05 \)) (Figure 4E).

**DISCUSSION**

The current findings definitely demonstrated that inadequate consumption of fruits may partly lead to an

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**Table 2: Qualification of fecal bacteria from BC patients and HCs by qPCR**

| Bacteria group              | HC (N = 16)     | BC (N = 26)     | \( p \) value |
|-----------------------------|-----------------|-----------------|---------------|
| **Domain Bacteria**         |                 |                 |               |
| **Phylum: Firmicutes**      |                 |                 |               |
| Clostridium cluster I       | 4.46 ± 0.77     | 3.96 ± 1.46     | 0.191         |
| Faecalibacterium prausnitzii| 7.41 ± 0.75     | 7.49 ± 0.97     | 0.775         |
| Clostridium cluster XI      | 4.97 ± 0.67     | 4.40 ± 0.85     | **0.031**     |
| Lactobacillus group         | 4.98 ± 0.90     | 4.83 ± 0.53     | 0.531         |
| Clostridium leptum subgroup  | 7.41 ± 1.17     | 6.82 ± 1.10     | 0.122         |
| Clostridium cocoides group  | 5.31 ± 0.89     | 5.56 ± 0.95     | 0.446         |
| **Phylum: Bacteroidetes**   |                 |                 |               |
| Bacteroides-Prevotella group| 7.54 ± 1.21     | 6.52 ± 0.90     | **0.004**     |
| Bacteroides fragilis group  | 5.94 ± 0.81     | 6.06 ± 1.17     | 0.732         |
| **Phylum: Actinobacteria**  |                 |                 |               |
| Bifidobacterium genus       | 2.80 ± 1.03     | 3.37 ± 1.12     | 0.100         |
| Atopobium cluster           | 5.88 ± 0.71     | 5.47 ± 0.77     | 0.128         |
| **Phylum: Proteobacteria**  |                 |                 |               |
| Enterobacteriaceae          | 3.04 ± 1.01     | 3.27 ± 1.03     | 0.580         |

The amounts of fecal bacteria from each group expressed as log_{10} bacteria per gram of stool. All values are presented as the mean ± SD. Significant differences were determined using ANOVA, and a \( p \) value less than 0.05 was considered as the statistical significance.
imbalance of the gut microbiota in BC. As a result, the intestinal mucosal integrity was damaged and followed by a cascade of inflammation-related reaction, which contributed to the carcinogenesis of BC.

Dietary factors and dietary patterns play a critical role in the modulation of the gut microbiota [23]. Dietary 24h-questionnaire showed that about 54% (14/26) BC patients consumed less than 20g fruits per day, which is far below the effective doses (200g/day) suggested before [32]. And possibly and consequently, the richness and composition of the intestinal flora were influenced. To our knowledge at the present, quite a few of population-investigations have showed the associations of gut microbiota and various disorders [33]. However, dietary factors and dietary patterns were ignored and rarely taken into consideration. The average level of fruit consumption in the Chinese population is very low [34]. Data from Chinese Society of Nutrition in 2012 showed that the average intake of dietary fiber is around 13g, far below the recommended adequate intake (AI) of 25g. And the inadequate intake of dietary fiber is associated with significantly lower overall mortality and also the prevalence of cancer [35, 36]. Our findings again provide the evidence that gut microbiota may function as a bridge between dietary factors and cancer. And larger-scale studies as well as the deep investigations should be undertaken in the future to address this issue.

Here, *Clostridium cluster XI*, known as a SCFA-producing microorganism, was significantly lower in BC patients than that in the HC group [37]. *Prevotella* is positively associated with dietary fiber consumption and induces the fermentation of complex polysaccharides

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**Figure 2**: The morphology of stage of BBN-induced bladder carcinogenesis and structural alterations of fecal microbiota in mice. (A) At the end of the study, the organs were removed and measured. The data are represented as mean ± SD. Statistical differences were determined using One-way ANOVA, and a p value of less than 0.05 was considered to be statistically significant. (B) The morphology of stage of BBN-induced bladder carcinogenesis in mice. Urinary bladders were processed for hematoxylin and eosin staining. Representative pictures are shown with 100× magnification, and 400× magnification. pTa, pT1, and pT2 refer to tumors that are noninvasive (superficial tumors); invade the lamina propria; and invade the muscularis propria, respectively. (C) PCR-DGGE fingerprinting of the fecal microbial community. Three samples collected randomly from each group (the samples labeled with number 1 to 3 belong to control mice, and samples with number 4 to 6 belong to BBN-induced mice (a). Cluster analysis according to the unweighted pair group method of arithmetic averages (UPGMA) (b). PCA based on the distance matrix (two-dimensional array) (c).
High level of *Prevotella* is commonly existed in healthy subjects but not those of patients with colorectal or breast cancer, which is consistent with our findings [40, 41]. Thus, the decreased quantities of *Clostridium* cluster XI and *Prevotella* in BC patients are unable to degrade these undigestible carbohydrates into the SCFAs.

The current findings showed that the consumptions of fruits were positively associated with the amounts of butyrate. Enough butyrate is essential for the growth and proliferation of the intestinal epithelial cells (IECs), and also keeps the homeostasis of intestinal mucosal barrier [26]. Consequently, the decreased butyrate in BC patients, due to the inadequate intake of fruits, might delay or even block the proliferation and development of IECs, and finally damaged the intestinal integrity.

Due to the poor intestinal integrity, LPS entered into the blood which stimulated the related immune cells or inflammatory cells to secret inflammatory cytokines. IL-6 is a representative pro-inflammatory cytokine. The increased IL-6 in serum was associated with metastasis and poor prognosis of cancer [42]. IL-6 activation in tumor microenvironment inhibits functional maturation of dendritic cells to activate effector T cells, blocking introduction of antitumor immunity in cancers [43]. IL-22 is produced by innate lymphoid cells, Th17 cells, and Th22 cells, particularly at mucosal surfaces [44].

![Figure 3: SCFAs concentrations in BC patients and BBN-induced BC mice.](image-url)

(A) Gas chromatography (GC) analysis of SCFAs concentrations in fecal samples from BC patients and HCs. (B) Relationship of butyric acid (μmol/g) and fruit intake (g/d) in the bladder cancer patients and healthy individuals. Correlation analysis performed with Pearson’s correlation, $r = 0.610$, and $p < 0.001$. (C) Gas chromatography (GC) analysis of SCFAs concentrations in the cecal contents from control and BBN-induced mice. Data are presented as the mean ± SD. Significant differences were determined using ANOVA, and a $p$ value less than 0.05 was considered as the statistical significance.
physiological state, IL-22 could induce the intestinal barrier regeneration and help to keep the homeostasis of the gut. However, IL-22 can also promote pathological inflammatory responses and carcinogenesis [45, 46]. SIgA is concentrated in the outer layer of intestinal mucus along with commensal bacteria and then is transported into external secretions [31]. SIgA represents the first line of antigen-specific immune defense in the gut lumen and keeps pro-inflammatory processes under control and preserves the integrity and functionality of the epithelial barrier [47]. Study has confirmed that disruption of mutualistic relationship between SIgA and the microbiota could lead to vicious cycle of decreasing SIgA and increasing dysbiosis over time [48]. In the current study, the level of SIgA was strongly elevated by nearly 2-fold along with imbalance of gut microbiota. Taken together, these cytokines are intensively secreted in response to LPS release induced by dysbiosis in gut, which in turns aggravated even initiated the carcinogenesis of bladder.

To our knowledge, it is the first time to reveal the gut microbiota-associated mechanism of bladder cancer. However, it should be noted that this study was carried out in a relatively small number of cases in all stages due to the low incidence of BC and the limiting ways to collect additional cases. Thus, additional larger-scale investigations should be performed in the future.

MATRERIALS AND METHODS

Patients and stool sample collection

All participants were informed about the study purpose during the admission interview, and voluntarily signed consent forms prior to enrolling in the study.

Figure 4: Excessive accumulation of LPS and D-lactic acid and inflammation in BC patients and BBN-induced mice due to deficiency of the gut barrier. (A) Concentrations of LPS and D-lactic acid in the BC patients and healthy controls. (B) Concentrations of LPS and D-lactic acid in the control and BBN-induced BC mice. (C) Representative hematoxylin and eosin staining of histological sections showed adenomatous changes in the colon (Figure 4C a-d) and cecum (Figure 4C e-h). Images at low magnification (100×) and one at higher magnification (400×) were obtained from the same section. (D) The expressions of zo-1, claudin-1 and occludin in mice were detected by quantitative real-time PCR. (E) Elevated concentrations of IL-6, IL-22 and SIgA in the control and BBN-induced BC mice. All values are presented as the mean ± SD. Significant differences were determined using ANOVA, and a p value less than 0.05 was considered as the statistical significance (*p <0.05, **p <0.01, ****p < 0.0001).
All procedures in this study were compliant with the Declaration of Helsinki, and the study protocol was approved by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. This trial was registered at www.chictr.org.cn as ChiCTR-OOC-16007937. BC patients were recruited for this study at the First Affiliated Hospital of Harbin Medical University between October 1, 2014 and January 30, 2015. HCs were recruited from patients who visited the First Affiliated Hospital of Harbin Medical University for physical examination. All of the BC patients and HCs were Han Chinese residents. None of the patients were undergoing radiotherapy, chemotherapy or other medical interventions. The exclusion criteria were as follows: 1) a history of taking antibiotics, aspirin, other non-steroidal anti-inflammatory drugs or probiotics within the previous 6 months before enrollment; 2) a vegetarian diet; and 3) a history of any other cancer or inflammatory disease of the intestine [49, 50]. All of the patients and healthy individuals had followed a relatively stable diet during the previous 5 years. According to the criteria, of the 74 subjects enrolled this study (40 for BC groups, 34 for HC group), a total of 26 newly diagnosed patients with histologically were finally confirmed BC and 16 HC were qualified.

Participants were provided waxed tissue paper (Epitope Diagnostics, CA, USA), which was laid down on the water in the toilet bowl prior to defecation to avoid contamination. All of the fresh stool samples were collected into sterilized and portable plastic containers and then were immediately placed on ice and transported to the laboratory. Aliquots (200 mg/ aliquot) were stored at −80°C until analysis.

Assessment of lifestyle factors and diet questionnaire

Data on the subjects’ demographics, lifestyle factors and dietary information were collected from questionnaires by 2 qualified clinical doctors. All the participants completed a questionnaire that was designed to provide information on health-related issues, including dietary information, smoking status, drinking status, and medical history [49, 51]. All of the participants were asked to recall the types and frequency of foods eaten in the past three months. Foods were grouped into three categories: fruits, vegetables and grains [49]. Individual food intake was computed from the reported consumption frequency of each specified unit of food based on data from the Chinese Society of Nutrition regarding the nutrient content in the specified food types. According to the standard for evaluation developed by Chen et al., scores were assigned for smoking status, daily alcohol intake, food intake (vegetables, grains and fruits) and frequency of weekly vigorous exercise [49] (Supplement 5).

Animal experimental design

Five-week-old male C57BL/6 mice (16–20 g in body weight, specific pathogen free) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. The mice were maintained at the Center of Safety and Evaluation of Drugs, Heilongjiang University of Chinese Medicine, throughout the entire procedure. Mice were allowed to acclimate for 1 week in a controlled environment (temperature 20 ± 2°C and humidity 55 ± 5% with a 12 h light/ dark cycle) and then were randomly assigned to two groups (10 mice for each group). Mice in the BC group received 0.05% (w/v) BBN (Sigma) in their drinking water for 12 weeks to induce bladder carcinogenesis according to the published literature [52, 53].

After treatment with BBN for 12 weeks, the mice were switched to normal drinking water for another 10 weeks. All animals were maintained in solid-bottom cages containing a bedding of softwood shavings and were allowed free access to food and water during the entire experimental procedure of 22 weeks. The whole experiment protocol was shown in Supplementary Figure 4. The animals’ body weight and consumption of food and water were monitored weekly during the entire experiment (Supplementary Figure 5).

The authors declared that all of the animal procedures were conducted in conformity with the guidelines of the Institutional Animal Care Use Committee, Heilongjiang Province, China. The protocol was approved by the Ethic Committee of Experimental Animals in Heilongjiang University of Chinese Medicine.

DNA extraction and 16S rRNA gene sequencing analysis

Total genomic DNA was extracted from fecal samples (human and mice, separately) aseptically using the QIAamp DNA Stool Mini Kit (Qiagen, CA). Pyrosequencing of the total genomic DNA extracted from human feces was carried out by using an Illumina HiSeq 2000 by Sangon Biotech Co. Ltd. (Shanghai, China) (Supplement 8). The sequences were grouped into unique OTUs using 97% identity thresholds. The richness, Shannon index and Simpson rarefaction plot were calculated to compare the microbial diversity and richness among the HCs and four subgroups of BC patients. A Venn diagram with shared OTUs was shown to depict the similarities and differences among the five groups. PCoA analysis was applied to compare the bacterial communities of the different groups based on phylogenetic information. The heatmap figure was generated using the R-package gplots [54].

PCR-DGGE analysis of fecal microbiota

Stool samples were randomly collected from mice prior to sacrifice. DNA was extracted using the QIAamp DNA extraction and 16S rRNA gene sequencing analysis
DNA Stool Mini Kit (Qiagen, CA). Bacterial 16S rDNA was amplified by PCR using the universal primers PRBA338f (5'CGC CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GAC TCC TAC GGG AGG CAG CAG 3') and reverse primer P518r (5'ATTA CCG CGG CTG CTGG 3') as previously described [55, 56]. PCA of the DGGE profiles was performed with Canoco for Windows [56, 57].

RNA extraction, cDNA synthesis and real-time quantitative PCR assay

RNA was extracted with TRIzol Reagent (Life Technologies), and cDNA was synthesized from total RNA using the gDNA Removal and cDNA Synthesis Supermix Kit (TransGen Biotech, Beijing, China). The copy number of target DNA for bacteria was determined by comparison with serially diluted standards of plasmid DNA (10² to 10⁷ copies) containing the respective amplicon for each set of primers and was run on the same plate under the same conditions [58]. Bacteria were quantified as log10 bacteria per gram of stool [59]. Target gene expression levels (zo-1, claudin-1 and occludin) were normalized to the internal control β-actin, and the relative expression levels of the target genes were calculated according to the 2^−ΔΔCT method [60]. The primer sequences were shown in Supplement 9.

Gas chromatography (GC) analysis of SCFAs content in fecal samples and cecal contents

Fecal samples (2 g) were homogenized with 10 mL of deionized water for 10 min and centrifuged at 13,200 g for 20 min at 4°C. The supernatant was immediately filtered through a 0.45μm microfiber filter, and then 1 mL of supernatant was placed in a 1.5mL GC vial with 100 μL of formic acid. Six SCFAs (≥99%, analytical standard, Sigma) were serially diluted to make standard curves separately. Then, the concentrations of six SCFAs were quantified by GC (Agilent 7890; Agilent Technologies, USA) equipped with a flame ionization detector (FID) according to the standards [61]. Total SCFAs concentrations were calculated as the sum of six SCFAs (acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid and iso-valeric acid). The concentrations of SCFAs in the cecum from BBN-treated mice were detected by GC. All values were shown as μmol/wet weight of stool (g).

Determination of LPS, D-lactic acid and SIgA levels in serum

For patients and healthy individuals, venous blood was collected. For mice, blood samples were collected by retro-orbital sinus puncture via the medial canthus of the eye using clean heparinized microhematocrit tubes [62]. The serum was centrifuged after clotting at room temperature. Aliquots of each serum sample were stored at ~80°C until analyzed. The concentrations of LPS, D-lactic acid and SIgA were determined by enzyme-linked immunosorbent assay (ELISA) kits from Jiancheng Biengineering Institute (Nanjing, P. R China) according to the manufacturer’s instructions. The concentrations were spectrophotometrically quantified by measuring the absorbance at 450 nm. The results were expressed as μmol/mL for D-lactic acid and ng/mL for LPS and SIgA.

Tissue collection, hematoxylin and eosin staining and scores

At the end of experiment, the mice were sacrificed, and the tissues (bladder, cecum and colon) were collected. Mice tissues were fixed in formalin, cut, and embedded on edge in cassettes. The slides were examined on an Olympus microscope. The tumor staging was recorded according to the criteria mentioned by Rex Munday et al. [63]. All the slides were grossly inspected in a blinded fashion by two pathologists from Harbin Medical University as previously described [56]. The cecum and colon slides were assigned an average histological score for 5 random fields and were scored from 0 to 4+ according to criteria, including inflammatory cells, goblet cells, mucosa thickening, submucosal cell infiltration, destruction of architecture, ulcer and crypt abscess (Supplement 10, 0 = normal, 1 to 4 = mild to severe) [64].

Multiple cytokine measurement with the MILLIPLEX MAP MICE Cytokines Panel®

For the quantitative analysis of cytokines (IL-6, IL-22, IL-1β, IL-10, IL-12p70, IL-17A, IL-23, TNF-α and IFN-γ), serum samples from mice were thawed and subjected to analysis on a Luminex 100 using the MILLIPLEX MAP MICE Cytokines Panel (Millipore, Billerica, MA, USA) according to the manufacturer’s recommended protocols.

Statistics analysis

All statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Continuous data were reported as the mean ± SD. One-way analysis of variance (ANOVA) was used to compare the differences. Categorical variables were compared using chi-squared and Fisher’s exact tests. Correlation between butyric acid and consumption of fruits were subjected to Pearson correlation analysis. A two-sided p value <0.05 was considered to indicate statistical significance.

Abbreviations

BBN: N-butyl-N-(4-hydrobutyl) nitrosamine; BC: bladder cancer; HC: healthy controls; LPS:
lipopolysaccharide; OTUs: operational taxonomic units; PCoA: UniFrac principal coordinates analysis; GC: gas chromatography; SCFAs: short-chain fatty acids; SIgA: secretory immunoglobulin A.

Author contributions

Yujuan Shan, Minghua Ren and Yongping Bao designed the research. Canxia He conducted the experiment and wrote the manuscript. Baolong Li took responsibility for the part of the animal experiment. Peijun Tian and Lei Huang analyzed the data. Xiaodong Liu collected the samples. Yongping Bao critically reviewed the manuscript content. All authors read and approved the final manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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