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Effects of prebiotics on immunologic indicators and intestinal microbiota structure in perioperative colorectal cancer patients

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ABSTRACT

Objective: The aim of the present study was to investigate the effects of prebiotics (containing fructooligosaccharides, xylooligosaccharides, polydextrose, and resistant dextrin) intake on immune function and intestinal microbiota structure in perioperative patients with colorectal cancer (CRC).

Methods: A randomized, double-blind, no-treatment parallel control clinical trial involving 140 perioperative patients (90 men and 50 women, aged 40–75 y) with CRC was performed. Patients were randomly divided into two groups: an intervention group (prebiotic group, $n = 70$) that received prebiotic supplementation of 30 g/d for 7 d, and a control group (non-prebiotic group, $n = 70$) that received no prebiotic supplementation. The nutritional and immunologic indices were evaluated for both groups before and after operation and analyzed against baseline values. Moreover, fecal samples were collected from 40 patients randomly chosen from the two groups to study intestinal microbiota, which was analyzed by sequencing the V3–V4 region of 16S ribosomal DNA using the Illumina (San Diego, CA) MiSeq (PE 2 × 300 bp) platform.

Results: Oral intake of prebiotics produced significant effects on immunologic indices in both the preoperative and postoperative periods, but the patterns of effects were different. In the preoperative period, prebiotics increased serum levels of immunoglobulin G (IgG; $P = 0.02$), IgM ($P = 0.00$), and transferrin ($P = 0.027$; all $P < 0.05$). In the postoperative period, enhanced levels of IgG ($P = 0.003$), IgA ($P = 0.007$), suppressor/cytotoxic T cells ($CD3^+CD8^+$; $P = 0.043$), and total B lymphocytes ($CD19^+$; $P = 0.012$) were identified in the prebiotic group (all $P < 0.05$). The differences in the intestinal microbiota at the phylum level were not statistically significant between the intervention and control groups ($P > 0.05$). At the genus level, prebiotics increased the abundance of *Bifidobacterium* ($P = 0.017$) and *Enterococcus* ($P = 0.02$; both $P < 0.05$) but decreased the abundance of *Bacteroides* ($P = 0.04$) in the preoperative period (all $P < 0.05$). In the postoperative period, the abundance of *Bacteroides* ($P = 0.04$) was decreased, but the abundance of *Enterococcus* ($P = 0.00$), *Bacillus* ($P = 0.01$), *Lactococcus* ($P = 0.00$), and *Streptococcus* ($P = 0.037$) increased in the non-prebiotic group (all $P < 0.05$); however, no significant change was identified in the abundance of *Enterococcus* ($P = 0.56$), *Lactococcus* ($P = 0.07$), and *Streptococcus* ($P = 0.56$) as a result of prebiotic intervention in this period (all $P > 0.05$). The abundance of *Escherichia-Shigella* was increased after prebiotic intake in the postoperative period ($P = 0.014$, $P < 0.05$). There was a notable trend of decline in the abundance of intestinal microbiota from preoperative to postoperative in the non-prebiotic group.

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Conclusions: Prebiotic intake is recommended to improve serum immunologic indicators in patients with CRC 7 d before operation. Prebiotics improved the abundance of four commensal microbiota containing opportunistic pathogens in patients with CRC. Surgical stress decreased the abundance of most intestinal microbiota in the intestinal tract but increased the abundance of some opportunistic pathogens and commensal microbiota. *Bacteroides* is a relevant bacterial species for further research on the mechanism of prebiotics.

Introduction

Prebiotics were first defined by Gibson and Roberfroid in 1995 [1]. The definition of a prebiotics was later updated in 2016 by the International Scientific Association for Probiotics and Prebiotics (ISAPP) as follows: a substrate that is selectively utilized by host microorganisms to confer health benefits [2]. Some prebiotics are non-digestible oligosaccharides; these include inulin-type fructans, xylooligosaccharides, and soy oligosaccharides [3]. Prebiotics alter the composition and activity of gut microbiota. They exert their functions through selective stimulation of growth of *Bifidobacteria* and other short-chain fatty acid (SCFA)–producing bacteria in colonic microbiota [4,5]. They can selectively induce the proliferation and activity of probiotic microorganisms [6,7]. Under normal physiological conditions, there is a dynamic intestinal microbiota-host balance. Disruption of this balance alters the intestinal microbiota, which is closely related to many diseases, including obesity, inflammatory bowel disease, adenoma, and even colorectal cancer (CRC) [8,9]. In a randomized controlled study, surgical patients treated with prebiotics combined with enteral nutrition support experienced shorter durations of hospital stay and had reduced levels of inflammatory cytokines than those who received enteral nutrition therapy alone [10]. Dogi et al. identified a probiotic strain that increased the level of immunoglobulin A (IgA) and B lymphocytes [11]. De Moreno de Leblanc et al. [12] found that tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2) producing cells, and CD4- and CD8-positive cell populations were enhanced in the lamina propria of the intestine of mice fed fermented milk containing probiotic bacteria. A phase III trial involving patients with CRC reported that preoperative oral administration of prebiotics promoted the recovery of intestinal function and improved nutritional status and immune function [13]. These studies suggested that prebiotics can improve the nutritional and immune function of the body. The prebiotic concept emerged from observations of selective stimulation of *Bifidobacterium* growth on inulin fermentation. To date, the use of sequencing approaches has allowed microbiota-wide assessment of relative abundance shifts. 16S rDNA microbiota profiling was used to assess the consequences of inulin fermentation in healthy adults. Vandeputte [14] reported the selective effect of inulin-type fructans on the human gut microbiota. The only genera consistently affected by inulin supplementation were *Bifidobacterium, Anaerostipes*, and *Bilophila*. This study found that prebiotics can improve the abundance of bacteria in the human gut. However, we are unaware whether prebiotics improve immune function while changing the intestinal microbiota structure, especially the structure of commensals in patients with CRC in the perioperative period using 16S ribosomal DNA (rDNA) microbiota sequencing approaches that have proven to be useful in a wide range of research areas.

Patients with CRC treated with radical surgery experience imbalance in their immune system and intestinal microbiota perioperatively. It is unclear whether colorectal radical surgery can significantly influence intestinal microbiota structure and whether the intestinal microbiota changes after tumor resection. Therefore we hypothesized that prebiotic supplement may improve immunologic indices and intestinal microbiota structure simultaneously in patients with CRC in the perioperative period. Colorectal radical surgery can significantly influence intestinal microbiota structure. The present study aimed to investigate the effects of prebiotics on the changes in the immunologic indices and intestinal microbiota structure during the entire perioperative period.

Materials and methods

Patients

A total of 140 patients (90 men and 50 women) with pathologically confirmed CRC who underwent radical resection were enrolled from the colorectal department of the General Hospital of Ningxia Medical University. The inclusion criteria were as follows: 1) age 40 to 75 years; 2) radiologic and pathologic diagnosis followed by radical resection for CRC; 3) absence of hydrothorax, ascites, dyscrasia, complete intestinal obstruction, cardiopulmonary dysfunction, and chronic wasting disease (e.g., diabetes); 4) lack or absence of radio- or chemotherapy before surgery; 5) no albumin and intestinal nutritional supplementation within 2 wk before surgery; 6) no metastasis before surgery; 7) no obvious hepatorenal dysfunction; 8) no congenital metabolic disease or gastrointestinal tract injury; 9) ability to stand, walk, comprehend, and answer; 10) no treatment with antibiotics within 6 mo before surgery; furthermore, patients who presented with postoperative infection and multiple-organ failure complications were excluded; 11) preoperative Nutrition Risk Screening 2002 score < 3; and 12) patients who had not consumed food containing prebiotics. All patients provided informed consent before their inclusion. The study was approved by the Medical Ethics Committee of the General Hospital of Ningxia Medical University (No. 2016-010; Clinical Trials Registration: http://www.chictr.org.cn/index.aspx, No. ChiCTR-IOR-17013269).

Study design

In this randomized, double-blinded, no-treatment parallel control, clinical trial study, randomization was performed using the block randomization procedure. The allocation sequence was randomly generated using a random allocation software. Randomization was performed by a third investigator with no clinical involvement in the trial to ensure the integrity of the blinding process. Patients were randomly divided into the non-prebiotic group (non-prebiotic, $n = 70$) and the prebiotic group (prebiotic, $n = 70$). The groups were further divided into pre- and postoperative subgroups (non-prebiotic/pre, non-prebiotic/post, prebiotic/pre, prebiotic/post groups). Serum was collected from all patients. Twenty patients randomly selected from each group contributed a total of 40 fecal samples that were analyzed by sequencing of the 16S rDNA V3–V4 regions to detect the intestinal microbiota structure by using the MiSeq (PE 2×300 bp) platform. Finally, 135 serum samples and 38 fecal samples were included and screened as part of the analysis (Fig. 1).

Patients in the intervention group (prebiotic group) also received a daily oral dose of 30 g prebiotic supplement (Hangzhou Niuqu Biotech Co., Hainengbo, China) containing fructooligosaccharide (25%), xylooligosaccharide (25%), polydextrose (25%), and resistant dextrin (25%) for 7 d from hospitalization to the day before the operation. We used free diet + prebiotics as the nutritional supplement in the preoperative period, and total parenteral nutrition was administered in the postoperative period. Free diet was supported by the hospital's department of nutrition. The dietary component of each patient was the same, and daily calorie intake was based on body mass index (BMI). Fecal samples were collected on the day before operation and 7 d after operation. Blood samples were collected 1 and 7 d before and after operation. The control group (non-prebiotic) received free diet without prebiotics in the preoperative and total parenteral nutrition in the postoperative period. Fecal and blood sample collection was performed in the same manner as that for the prebiotic group. Total parenteral nutrition was administered after surgery (20 kcal/kg calorie per day) using the same parenteral nutritional components in two groups, which included medium- and long-chain fat emulsion, compound amino acids, electrolytes, and glucose. Calorie nitrogen ratios were controlled at 1:100. The same second-generation cephalosporin was

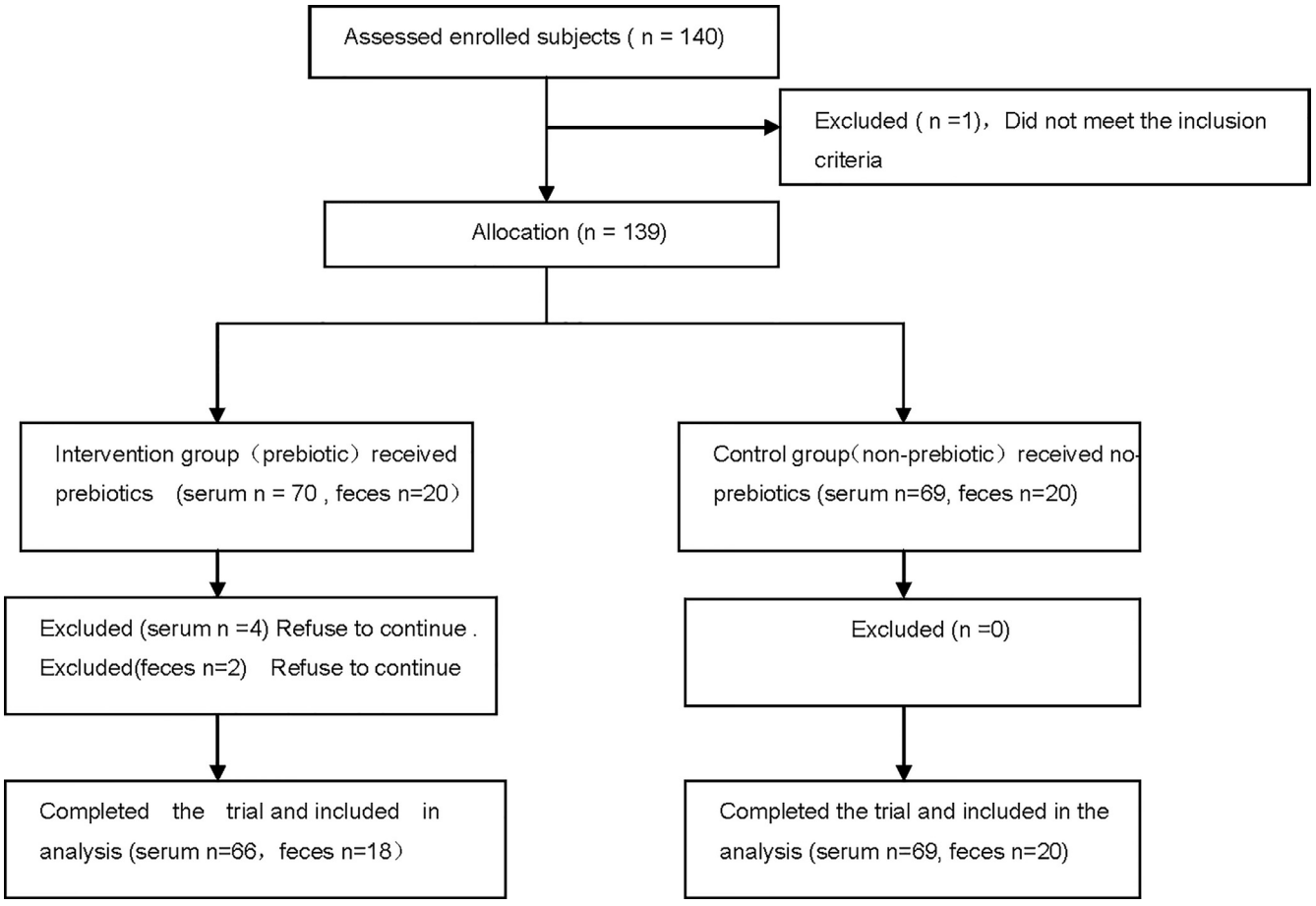

Fig. 1. Flow diagram of patient recruitment in the study. A total of 140 patients were initially selected for inclusion in the trial. Finally, 135 patients were selected after screening. The serum samples of these patients were included in the experiment according to the grouping. Thirty-eight fecal samples were randomly selected for 16S rDNA detection. Prebiotic, prebiotic group; non-prebiotic, non-prebiotic group.

administered within 48 h intravenously after surgery to prevent infection in the two groups.

Anthropometric assessments

Patient characteristics, including age, sex, BMI, and diagnosis, were recorded. Body weight and height were measured with a calibrated digital scale and stadiometer, respectively. BMI was calculated as weight (kg) divided by height (m) squared.

Biochemical assays

Patients' biochemical indices were investigated using the serum samples collected on the first day of admission and postoperatively and on the day before surgery and 7 d postoperatively. Blood samples were collected in the morning after overnight fasting for 12 h and the serum was separated by centrifugation at 2000 rpm for 10 min (HEMR, China) before detection. Serum hemoglobin and lymphocytes were measured by an automatic hematology analyzer (Sysmex, Kobe, Japan) using sodium lauryl sulfate hemoglobin detection and flow cytometry. Serum albumin was measured by an automatic biochemical analyzer (Siemens, Munich, Germany) using a chemical assay. Prealbumin, transferrin, IgA, IgG, and IgM levels were measured by the BN II automatic plasma protein analyzer (Siemens) by using immunoturbidimetry. Cellular immune indices, including total T lymphocytes ($CD3^+$), helper inducer T cells ($CD3^+CD4^+$), suppressor/cytotoxic T cells ($CD3^+CD8^+$), natural killer cells ($CD16^+CD56^+$), and total B lymphocytes ($CD19^+$) were determined by BD-FACS Canto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ) by using immunofluorescence cell staining. Blood and fecal samples were collected before intestinal preparation. The primary outcomes reported in this study were humoral immunity indices (IgA, IgG, and IgM) and cellular immune indices. The other indices were reported as secondary outcome.

Fecal assays

Intestinal microbiota structure was analyzed by sequencing the V3–V4 region of 16S rDNA isolated from fecal samples by using MiSeq combined with a multivariate statistical analysis. Fresh fecal samples were preserved at −80°C in the biobank of the General Hospital at Ningxia Medical University. A physicochemical method was used for DNA extraction to prevent the effects of different reagents. Polymerase chain reaction (PCR) testing was performed according to the following criteria: consistent dose, controlled minimum cycle number, consistent amplification cycle number, and standard procedure of Illumina MiSeq PE300 library construction and sequencing. This experiment consisted of DNA extraction and quality control, PCR amplification of the V3–V4 region of 16S rRNA genes, product purification, DNA library construction, and MiSeq sequencing.

The 38 fecal samples were subjected to identical procedures for DNA extraction and PCR amplification by the same laboratory staff. Samples were suspended in 790 μL of sterile lysis buffer (4M guanidine thiocyanate, 10% N-lauroyl sarcosine, and 5% N-lauroyl sarcosine–0.1 M phosphate buffer [pH 8.0]) in 2 mL screw-cap tubes containing 1 g of glass beads (0.1 mm BioSpec Products, Inc., Bartlesville, OK). This mixture was vortexed vigorously and then incubated at 70°C for 1 h. The DNA was extracted after lysis following the manufacturer's instructions for bacterial DNA extraction by using the E.Z.N.A. Stool DNA Kit (Omega Bio-Tek, Norcross, GA) and stored at −20°C for further analysis. The extracted DNA from each sample was used as a template to amplify the V3–V4 region of 16S

rDNA genes. The primers F1 and R2 (5′ - CCTACGGGNGGCWGCAG-3′ and 5′- GACTACHVGGGTATCTAATCC-3′), which correspond to positions 341 to 805 in the *Escherichia coli* 16S rDNA gene, were used to amplify the V3–V4 region of each sample by PCR. PCR reactions were performed using an EasyCycler 96 PCR system (Analytik Jena Corp., Jena, Germany) under the following conditions: 3 min of denaturation at 95°C followed by 21 cycles of 0.5 min at 94°C (denaturation), 0.5 min for annealing at 58°C, and 0.5 min at 72°C for elongation, with a final extension at 72°C for 5 min. The products from different samples were indexed and mixed in equal ratios for sequencing by Shanghai Itechgene Technology Co. Ltd. using the MiSeq platform (Illumina) according to the manufacturer's instructions.

Bioinformatics analyses

Bioinformatics analyses of fecal samples were performed as follows after sequencing the 16S rDNA V3–V4 regions on a MiSeq platform (PE2 × 300 bp). Clean data were extracted from raw data using USEARCH 8.0 with the following criteria: 1) sequences of each sample were extracted using each index with zero mismatch; 2) sequences with overlap < 50 bp were discarded; 3) sequences with overlap error rates > 0.1 were discarded; and 4) sequences < 400 bp after merging were discarded. Quality-filtered sequences were clustered into unique sequences and sorted in the order of decreasing abundance to identify representative sequences by using Uparse, according to the Uparse Operational Taxonomic Unit (OTU) analysis pipeline. Singletons were omitted in this step. OTUs were classified on the basis of 97% similarity after chimeric sequences were removed using Uparse (version 7.1; http://drive5.com/uparse/). The phylogenetic affiliation of each 16S rDNA gene sequence was analyzed using the RDP Classifier (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rDNA database by using a confidence threshold of 70%. Sample diversity metrics were assessed on the basis of the nonparametric Shannon-Wiener and Simpson diversity indices. The non-parametric Mann-Whitney *U* test was used to test for significant differences between the four groups. Comparison of multiple groups was performed using the nonparametric Kruskal-Wallis test. Both weighted and unweighted UniFrac were calculated in QIIME. The QIIME pipeline was also used to generate principal coordinate analysis plots and to visualize the unweighted UniFrac dissimilarity. Permutational multivariate analysis of variance was used to test for statistical significance between the groups by using 10,000 permutations (QIIME package). The 16S rDNA gene sequence information in this study has been submitted to the GenBank Sequence Read Archive database under accession number SRP127592.

Statistical analyses

Patient data and blood indices were analyzed using SPSS 24.0 software (IBM Corp., Armonk, NY). Quantitative data are presented as mean ± standard deviation, and qualitative data are presented as percentages. The χ^2 test was used to identify ratio differences between two groups. Differences among groups were analyzed using analyses of variance. A paired *t* test was used for before and after intervention comparisons. Analysis of covariance was used to identify any differences between two groups after surgical stimulation by adjusting for the confounding factors of baseline indices of the parameters. The Kolmogorov-Smirnov test was used to assess the normality of data. *P* values < 0.05 were considered statistically significant.

Table 1
General characteristics of patients in the two groups before surgery

Variable (sex %)	Prebiotic group (n = 66)	Non-prebiotic group (n = 69)	*P*
Male*	n = 44 (66.7%)	n = 43 (62.3%)	0.36
Female*	n = 22 (33.3%)	n = 26 (37.7%)	
Age†	62.62 ± 9.627	60.29 ± 9.54	0.16
BMI†	23.84 ± 3.37	23.63 ± 3.48	0.72

BMI, body mass index.
P values < 0.05 were considered statistically significant.
*P values for χ^2 test.
†P values for analysis of variance. Data are presented as mean ± standard deviation.

Results

General patient characteristics

Table 1 summarizes the general characteristics of patients in both groups before surgery. There were no significant differences in age (P = 0.16), sex (P 036), and BMI (P = 0.72) between the two groups (all $P > 0.05$).

Nutritional and immunologic indices

No significant baseline differences were identified between the prebiotic and non-prebiotic groups (all $P > 0.05$; Table 2). Supplementation with prebiotics significantly increased transferrin (P = 0.027), IgG (P = 0.02), and IgM (P = 0.00) levels preoperatively (all $P < 0.05$). Postoperatively, IgG (P = 0.003), IgA (P = 0.007), $CD3^+CD8^+$ (P = 0.043), and $CD19^+$ (P = 0.012) levels were significantly elevated in the prebiotic group (all $P < 0.05$).

Effects of prebiotics and operation on the distribution and diversity of intestinal microbiota at the phylum and genus levels

The Shannon-Wiener diversity index (Fig. 2a) revealed an abundant microbial diversity with prebiotic supplementation (non-prebiotic/pre versus prebiotic/pre and non-prebiotic/post versus prebiotic/post) and a decline in microbial diversity postoperatively (non-prebiotic/pre versus non-prebiotic/post and prebiotic/pre versus prebiotic/post). Principal component analysis (PCA) (Fig. 2b) indicated that the non-prebiotic/pre and prebiotic/pre groups were enriched in the left region, whereas the non-prebiotic/post and prebiotic/post groups were enriched in the right region, with a significant tendency of dispersion.

The relative abundance of each species was calculated by the Wilcoxon test at the phylum and genus levels. As shown in Figure 2c and Table 3, the dominant bacterial communities of the phylum level included *Bacteroidetes, Firmicutes, Proteobacteria,* and *Actinomycetes* in different groups. Preoperatively, *Bacteroidetes* was dominant (41%) in the non-prebiotic group, whereas *Firmicutes* was dominant (51%) in the prebiotic group. The abundance of *Bacteroidetes* decreased from 41% (non-prebiotic/pre) to 33% (prebiotic/pre), whereas the abundance of *Firmicutes, Proteobacteria*, and *Actinomycetes* increased from 39% (non-prebiotic/pre) to 51% (prebiotic/pre), from 10% (non-prebiotic/pre) to 11% (prebiotic/pre), and from 0.4% (non-prebiotic/pre) to 3% (prebiotic/pre), respectively. Postoperatively, the abundance of *Bacteroidetes* was unchanged in both groups. The abundance of *Firmicutes* decreased from 53% to 32%, but the abundance of *Proteobacteria* and *Actinomycetes* increased from 23% to 41% in the prebiotic group and from 1% to 2% in the non-prebiotic group. These differences were not statistically significant (P>0.05).

Table 2
Biochemical indicators in patients before and after surgery

Biochemical indicators before and after surgery	Preoperative Non-prebiotic	Prebiotic	P^*	Postoperative Non-prebiotic	Prebiotic	P^*
Hgb (g/L)						
Before[‡]	130.75±20.77	127.67±22.21	0.406	110.86 ± 16.939	109.26 ± 14.30	0.556
After[§]	130.42 ± 20.894	125.92 ± 22.989	0.236	116.03 ± 17.862	112.67 ± 15.281	0.243
$P^†$	0.925	0.659		0.08	0.188	
ALB(g/L)						
Before[‡]	37.50 ± 4.00	36.50 ± 3.68	0.133	28.383 ± 2.96	29.59 ± 4.71	0.075
After[§]	37.457 ± 3.984	37.047 ± 3.986	0.552	30.999 ± 5.330	31.083 ± 4.963	0.924
$P^†$	0.954	0.41		0.001	0.079	
LYM ($\times 10^9$/L)						
Before[‡]	1.87 ± 0.60	1.80 ± 0.57	0.492	1.2 ± 0.53	1.22 ± 0.42	0.785
After[§]	1.878 ± 0.597	1.942 ± 0.483	0.490	1.35 ± 0.51	1.2133 ± 0.443	0.211
$P^†$	0.988	0.145		0.077	0.943	
PAB (g/L)						
Before[‡]	0.28 ± 0.64	0.27 ± 0.08	0.568	0.178 ± 0.066	0.191 ± 0.07	0.264
After[§]	0.277 ± 0.064	0.266 ± 0.083	0.391	0.173 ± 0.074	0.192 ± 0.059	0.095
$P^†$	0.99	0.785		0.053	0.915	
TRF (g/L)						
Before[‡]	2.27 ± 0.48	2.49 ± 0.60	0.24	1.83 ± 0.46	1.96 ± 0.60	0.165
After[§]	2.266 ± 0.482	2.70 ± 0.46	0.007	1.59 ± 0.558	1.93 ± 0.587	0.296
$P^†$	0.924	0.027		0.057	0.812	
IgG (g/L)						
Before[‡]	11.66 ± 1.49	11.98 ± 1.52	0.213	8.39 ± 1.67	10.07 ± 1.41	0.00
After[§]	11.679 ± 1.491	12.271 ± 1.599	0.027	9.053 ± 2.366	10.643 ± 1.542	0.003
$P^†$	0.935	0.02		0.003	0.028	
IgA (g/L)						
Before[‡]	2.35 ± 0.89	2.15 ± 0.83	0.178	1.59 ± 0.76	1.91 ± 0.57	0.007
After[§]	2.344 ± 0.894	2.2432 ± 0.551	0.432	1.778 ± 0.954	2.0309 ± 0.637	0.074
$P^†$	0.966	0.449		0.03	0.242	
IgM (g/L)						
Before[‡]	0.69 ± 0.32	0.70 ± 0.31	0.951	0.50 ± 0.31	0.94 ± 0.47	0.002
After[§]	0.705 ± 0.333	1.1518 ± 0.439	0.00	0.628 ± 0.417	1.034 ± 0.496	0.831
$P^†$	0.783	0.00		0.42	0.281	
$CD3^+$ (%)						
Before[‡]	69.36 ± 8.94	69.25 ± 9.89	0.947	70.00 ± 8.93	71.82 ± 8.78	0.236
After[§]	69.351 ± 8.921	68.635 ± 9.767	0.657	70.593 ± 8.772	72.594 ± 8.971	0.192
$P^†$	0.994	0.781		0.696	0.617	
$CD3^+CD8^+$ (%)						
Before[‡]	30.15 ± 8.47	29.83 ± 7.90	0.825	26.86 ± 7.51	28.11 ± 7.30	0.33
After[§]	29.65 ± 8.323	30.95 ± 7.425	0.341	26.63 ± 8.005	29.33 ± 7.303	0.043
$P^†$	0.729	0.415		0.861	0.341	
$CD3C^+D4^+$ (%)						
Before[‡]	40.70 ± 7.24	41.23 ± 7.04	0.669	40.94 ± 7.74	41.91 ± 7.77	0.466
After[§]	40.582 ± 7.376	40.123 ± 7.537	0.721	41.478 ± 7.601	42.479 ± 7.676	0.448
$P^†$	0.923	0.384		0.68	0.675	
$CD16^+CD56^+$ (%)						
Before[‡]	19.74 ± 9.16	17.96 ± 9.68	0.274	19.51 ± 10.43	18.71 ± 12.08	0.68
After[§]	19.590 ± 9.240	20.098 ± 11.3802	0.776	19.665 ± 10.646	18.697 ± 12.230	0.624
$P^†$	0.924	0.247		0.932	0.995	
$CD19^+$ (%)						
Before[‡]	13.22 ± 4.97	12.74 ± 4.25	0.547	12.89 ± 7.57	18.42 ± 11.63	0.001
After[§]	13.057 ± 4.932	11.721 ± 3.670	0.078	14.230 ± 7.873	18.572 ± 11.735	0.012
$P^†$	0.847	0.144		0.31	0.941	

ALB, albumin; $CD3^+$, total T lymphocytes; $CD3^+CD4^+$, helper inducer T cells; $CD3^+CD8^+$, suppressor/cytotoxic T cells; $CD16^+CD56^+$, natural killer cells; $CD19^+$, total B lymphocytes; Hgb, hemoglobin; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; LYM, lymphocyte; PAB; prealbumin; TRF, transferrin.
Data are presented as mean ± SD. P values < 0.05 were considered statistically significant.
*P values for analysis of covariance, with postoperative adjustment for the confounding effects of baseline indices of parameters.
†P values for paired t test; $P < 0.05$ was statistically significant.
‡Data are baseline indices preoperatively and postoperatively. The indices were collected on the first day of admission and the first day postoperatively.
§The indices were collected 7 d after admission preoperatively and 7 d postoperatively, respectively.

Figure 2d summarizes the distribution of the relatively dominant bacteria at the genus level. Intestinal microbiota varied between the four groups. The differences in the related abundance presented in Table 4 were measured using the Wilcoxon test. *Bacteroides* was significantly decreased in the prebiotic/pre group (P = 0.04). The abundance of *Bifidobacterium* was increased significantly in the prebiotic/pre group (P = 0.017) and prebiotic/post group (P = 0.025). The abundance of *Enterococcus* was significantly increased in the prebiotic/pre group (P = 0.02), with the opposite result observed in the prebiotic/post group (P = 0.021). The abundance of *Bacteroides* was significantly decreased, whereas that of *Enterococcus* (P = 0.00), *Bacillus* (P = 0.00), *Lactococcus* (P = 0.00), and *Streptococcus* (P = 0.037) was increased in the non-prebiotic group postoperatively (Table 5). However, there were no changes in the prebiotic group postoperatively, except for *Bacillus* (P = 0.03) and *Escherichia-Shigella* (P = 0.03).

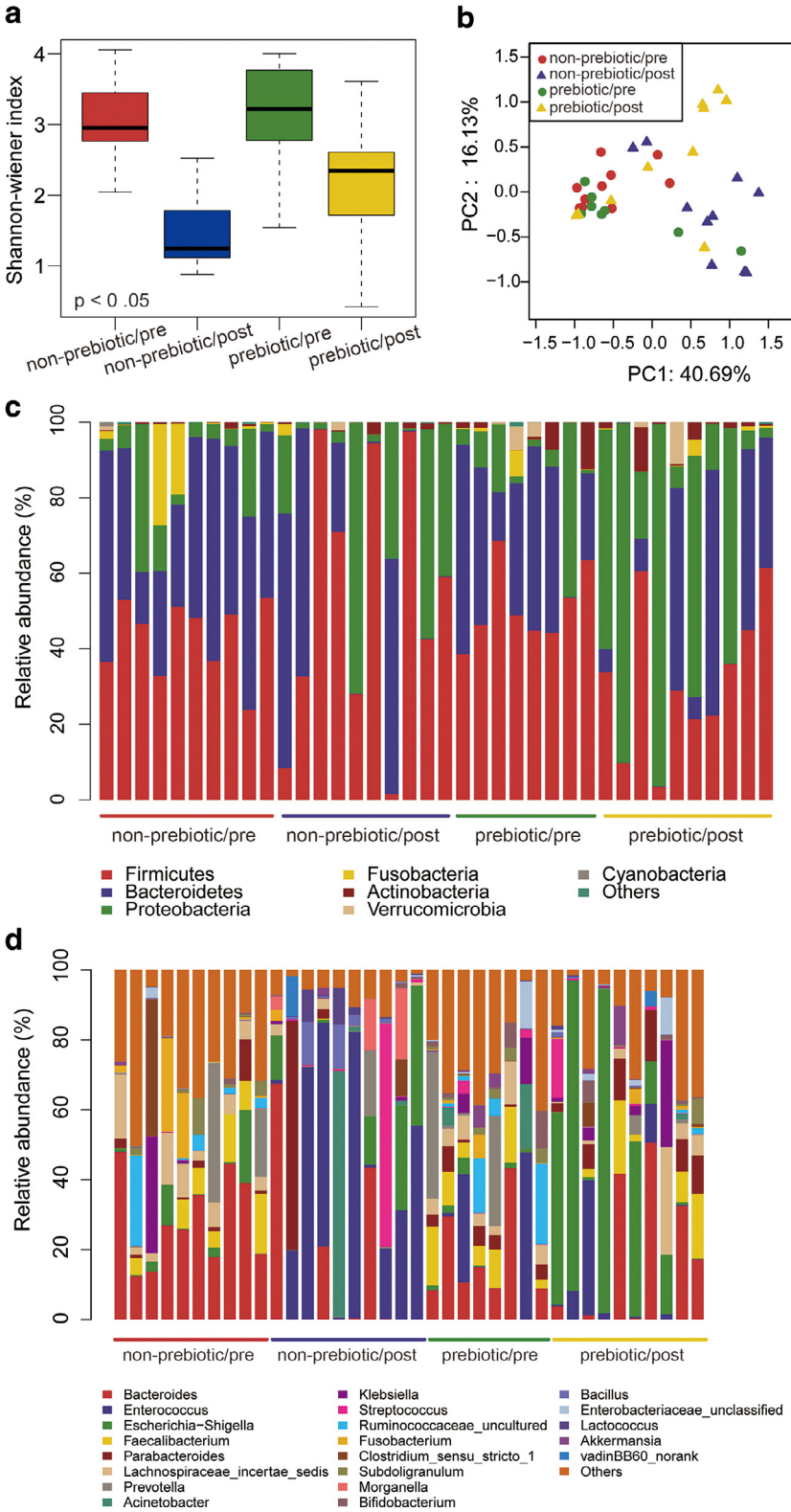

Fig. 2. Dominant microbial species at the phylum and genus levels. Non-prebiotic/pre (red), non-prebiotic group/preoperative; non-prebiotic/post (blue), non-prebiotic group/postoperative; prebiotic/pre (green), prebiotic group/preoperative; prebiotic/post (yellow): prebiotic group/postoperative. (a) The microbiota diversity in the intervention group increased after supplementation of prebiotics (non-prebiotic/pre [red] versus prebiotic/pre [green] and non-prebiotic/post [blue] versus prebiotic/post [yellow]). Surgical stress led to reduction in intestinal microbiota diversity (non-prebiotic/pre [red] versus non-prebiotic/post [blue] and prebiotic/pre [green] versus prebiotic/post [yellow]. (b) The first principal component representing the surgical factor (x, PC1) induced an obvious change in microbial communities, with a contribution rate of 40.72%. The second principal component representing the prebiotics intake factor (y, PC2) also changed the intestinal microbiota with a contribution rate of 16.13%. (c) *Firmicutes* (red), *Bacteroidetes* (blue), and *Proteobacteria* (green) were the dominant microbiota at the phylum level. The intake of prebiotics did not affect the microbiota significantly at the phylum level ($P > 0.05$). (d) The abundance of *Bacteroides* (red) was decreased ($P < 0.05$), and the abundance of *Bifidobacterium* (brown) and *Enterococcus* (blue) was increased after prebiotic intake in patients with colorectal cancer ($P < 0.05$).

Table 3
Changes in the dominant microbial species at the phylum level

	Preoperative					Postoperative				
	Non-prebiotic		Prebiotic			Non-prebiotic		Prebiotic		
Phylum	Relative contribution %*	Mean ± SD %	Relative contribution %*	Mean ± SD %	*P*†	Relative contribution %*	Mean ± SD %	Relative contribution %*	Mean ± SD %	*P*†
Bacteroidetes	0.41	41.17 ± 0.14	0.33	32.59 ± 0.19	0.26	0.22	22.02 ± 0.36	0.22	22.19 ± 0.25	0.85
Firmicutes	0.43	43.11 ± 0.10	0.51	51.08 ± 0.10	0.3	0.53	53.3 ± 0.36	0.32	32.26 ± 0.19	0.23
Proteobacteria	0.1	10.03 ± 0.12	0.11	10.84 ± 0.15	0.75	0.23	23.3 ± 0.26	0.41	36.61 ± 0.12	0.12
Actinomycetes	0.004	0.004 ± 0.004	0.03	0.029 ± 0.045	0.26	0.01	0.7 ± 0.01	0.02	2.27 ± 0.035	0.06

SD, standard deviation.
*Relative contribution of a genus in the non-prebiotic or prebiotic group was calculated as the percentage of the sequences of this genus to all sequences identified pre- and postoperatively.
†$P < 0.05$ was considered statistically significant.

OTU clusters and relative abundances of dominant microbial species

There was a notable decline in the abundance of intestinal microbiota preoperatively compared with postoperatively (Fig. 3). In addition, comparison of the non-prebiotic/pre and non-prebiotic/post data revealed an increase in the abundance of *Lactococcus* OTU21, *Enterococcus* OTU90, *Streptococcus* OTU82, and *Bacillus* OTU20 (yellow boxes in Fig. 3) and a decline in the abundance of *Bacteroides* (red boxes in Fig. 3). The abundance of *Escherichia-Shigella OTU107* (blue box in Fig. 3) indicated an obvious increase in the prebiotic group postoperatively. The preoperative intake of prebiotic caused an increase in *Bifidobacterium* OTU197 and *Enterococcus* OTU90 (yellow boxes in Fig. 3) and a decline in *Bacteroides* OTU224 (red boxes in Fig. 3). The results coincided with the results at the genus level.

Discussion

The major finding of the current investigation, summarized in Table 2, is that prebiotic supplementation significantly increased IgG and IgM levels preoperatively. Postoperatively the supplementation enhanced the levels of IgG, IgA, total B lymphocytes ($CD19^+$), and suppressor/cytotoxic T cells ($CD3^+CD8^+$). Oligofructose can improve immune system functions by the selective stimulation of the growth of *Bifidobacteria* and other short-chain fatty acid–producing bacteria in the colonic microbiota [15]. Prebiotic fibers are common prebiotics consumed by probiotic microorganisms for energy metabolism and growth [16–18]. Xu et al. [19] reported that soluble dietary fiber (extracted from fruits and vegetables) significantly enhanced the levels of $CD4^+$ cells, IgA, and IgM 7 d after radical resection in patients with colon cancer. We also found similar results. Prebiotic intervention has been found to have a positive effect on the immune status of surgical CRC patients. IgG is the most abundant and important protein among human immunoglobulins. It protects the body from infection by binding to a variety of pathogens that include bacteria and viruses. Miyatani et al. [20] found that serum IgG4 levels were significantly lower in patients with advanced gastric cancer than in healthy controls. Of the four subclasses of IgG in humans (IgG1–4) [21], there is accumulating evidence [22] of IgG4 and IgG4-positive plasma cells in several cancers, including pancreatic cancer [23], cholangiocarcinoma [24], and gastric cancer, and the association of IgG4 and IgG4-positive plasma cells with patient prognoses has been reported. In our study, IgG levels in patients were increased after prebiotic supplementation pre- and postoperatively (Table 2). Further investigation is required to determine whether IgG is involved in the pathogenesis of CRC. Transferrin is the major transport protein of iron in the body. The protein is mainly synthesized in the liver. Iron deficiency is associated with increased transferrin in the body [25]. Transferrin has been found to decrease in inflammation, cancers, and certain diseases [26]. The efficacy of prebiotics on reducing inflammatory cytokines and increasing antiinflammatory cytokines was confirmed [6]. Inflammatory factors were found to be increased in most patients with CRC. Therefore a possible reason that prebiotics increase the level of transferrin is that prebiotics relieve the inflammatory reaction of the body, resulting in increased transferrin level.

Although we found alterations of the intestinal microbial community at the phylum level, these differences were not statistically significant. Significance was apparent at the genus level. Oral prebiotic intake led to a decline of *Bacteroidetes* in the prebiotic/pre group. The abundance of *Bifidobacterium* and *Enterococcus* increased significantly in the prebiotic/pre group. A study [15] reported the significantly greater abundance of *Enterococcus, Streptococcus,* and *Bacteroides* in advanced colorectal adenoma (A-CRA) patients than in healthy individuals. Although we did not include healthy individuals, we found a reduction in the abundance of *Bacteroides* as commensals after prebiotic supplementation, with a further decline in *Bacteroides* after colon cancer surgical resection. *Bacteroides* are normally mutualistic organisms that make up the most substantial portion of the mammalian gastrointestinal microbiota [27]. *Bacteroides* also benefit their host by inhibiting potential pathogens from colonizing the gut. Some species (e.g., *Bacteroides fragilis*) are opportunistic human pathogens. A strain of *B.* fragilis called enterotoxigenic *B. fragilis* reportedly triggers cellular proliferation and colonic colitis and strongly induces colonic tumors in multiple intestinal neoplasia mice [28]. Recently, accumulating evidence has substantiated the association between *B. fragilis* toxin and colorectal pathogenesis [29,30]. The reason for the reduction of abundance of *Bacteroides* by prebiotics is not clear and needs further study. Prebiotics increased the abundance of *Bifidobacteria* at the genus level. *Bifidobacteria* are closely related to gut health in humans and animals [31,32]. These bacteria are the first colonizers of the newborn infant gut and further develop into abundant communities [33]. Turroni et al. [34] found that *Bifidobacterium* can induce cytokine production, promote B-cell growth, and regulate immune function. In addition, animal studies have found that *Bifidobacteria* can activate mouse peritoneal macrophages, thereby enhancing IL-1 activity and IL-6 production [35]. It is proposed that *Bifidobacteria* prevent against CRC by regulating intestinal microbiota, enhancing host immune response, and degrading potential carcinogens [35,36].

Important clinical infections caused by *Enterococcus* include urinary tract infections, bacteremia, bacterial endocarditis, diverticulitis, and meningitis [37]. As an opportunistic pathogen, *Enterococcus* usually

Table 4
Differences in the dominant microbial species at the genus level in the non-prebiotic and prebiotic groups

Phylum	Genus	Preoperatively						Postoperatively					
		Non-prebiotic		Prebiotic		$P^{\dagger}$	Tendency in the two groups[‡]	Non-prebiotic		Prebiotic		$P^{\dagger}$	Tendency in the two groups[‡]
		Relative contribution %[*]	Mean ± SD %	Relative contribution %[*]	Mean ± SD %			Relative contribution %[*]	Mean ± SD %	Relative contribution %[*]	Mean ± SD %		
Bacteroidetes	*Bacteroides*	28.25	28 ± 0.13	15.57	16 ± 0.140	0.04	↓	13.27	13.26 ± 0.238	14.75	14.7 ± 0.20	0.79	
Actinomycetes	*Bifidobacterium*	0.35	0.3 ± 0.5	2.64	2.6 ± 0.040	0.017	↑	0.23	0.22 ± 0.0048	1.05	1.04 ± 0.11	0.025	↑
Firmicutes	*Enterococcus*	0.02	0.02 ± 0.002	9.99	9.99 ± 0.180	0.02	↑	34.69	34.7 ± 0.310	6.24	6.24 ± 0.12	0.021	↓
Firmicutes	*Lactococcus*	0.02	0.03 ± 0.003	0.05	0.04 ± 0.001	0.374		2.36	2.3 ± 0.040	0.22	0.2 ± 0.003	0.75	
Firmicutes	*Streptococcus*	0.1	0.1 ± 0.007	0.84	0.8 ± 0.010	0.23		6.66	6.65 ± 0.20	2	2.0 ± 0.05	0.62	
Firmicutes	*Bacillus*	0.02	0.020 ± 0.003	0.05	0.05 ± 0.001	0.62		3.09	3.08 ± 0.050	0.26	0.2 ± 0.004	0.064	
Proteobacteria	*Escherichia-Shigella*	4.01	4.02 ± 0.068	1.44	1.4 ± 0.015	0.69		9.8	9.8 ± 0.140	31.8	32 ± 0.37	0.104	

SD, standard deviation.
*Relative contribution of genus in non-prebiotic or prebiotic patients was calculated as the percentage of the sequences of this genus to all sequences observed pre- and postoperatively.
†$P < 0.05$ was considered statistically significant.
‡The ↓ means decrease and ↑ means increase, with a tendency of statistical significance.

Table 5
Differences in the dominant microbial species at the genus level pre- and postoperatively

Phylum	Genus	Non-prebiotic						Prebiotic					
		Preoperatively		Postoperatively		$P^{\dagger}$	Tendency in the two groups[‡]	Preoperatively		Postoperatively		$P^{\dagger}$	Tendency in the two groups[‡]
		Relative contribution %[*]	Mean ± SD %	Relative contribution %[*]	Mean ± SD %			Relative contribution %[*]	Mean ± SD %	Relative contribution %[*]	Mean ± SD %		
Bacteroidetes	*Bacteroides*	28.25	28 ± 0.13	13.27	13.26 ± 0.24	0.04	↓	15.57	16 ± 0.140	14.75	14.7 ± 0.20	0.50	
Actinomycetes	*Bifidobacterium*	0.35	0.3 ± 0.5	0.22	0.23 ± 0.004	0.47		2.64	2.6 ± 0.040	1.05	1.04 ± 0.11	0.82	
Firmicutes	*Enterococcus*	0.02	0.02 ± 0.002	34.70	34.7 ± 0.313	0.00	↑	9.99	9.99 ± 0.180	6.24	6.24 ± 0.12	0.56	
Firmicutes	*Lactococcus*	0.02	0.03 ± 0.003	2.40	2.4 ± 0.04	0.00	↑	0.05	0.04 ± 0.001	0.22	0.2 ± 0.003	0.07	
Firmicutes	*Streptococcus*	0.1	0.1 ± 0.007	6.65	6.65 ± 0.20	0.037	↑	0.84	0.8 ± 0.010	2	2.0 ± 0.05	0.56	
Firmicutes	*Bacillus*	0.02	0.020 ± 0.003	3.08	3.08 ± 0.50	0.01	↑	0.05	0.05 ± 0.001	0.26	0.2 ± 0.004	0.03	↑
Proteobacteria	*Escherichia-Shigella*	4.01	4.02 ± 0.068	9.80	9.8 ± 0.14	0.62		1.44	1.4 ± 0.015	31.8	32 ± 0.37	0.014	↑

SD, standard deviation.
*Relative contribution of a genus pre- or postoperatively was calculated as the percentage of the sequences of this genus to all sequences in the non-prebiotic and prebiotic groups.
†$P < 0.05$ was considered statistically significant.
‡The ↓ means decrease and ↑ means increase, with a tendency of statistical significance.

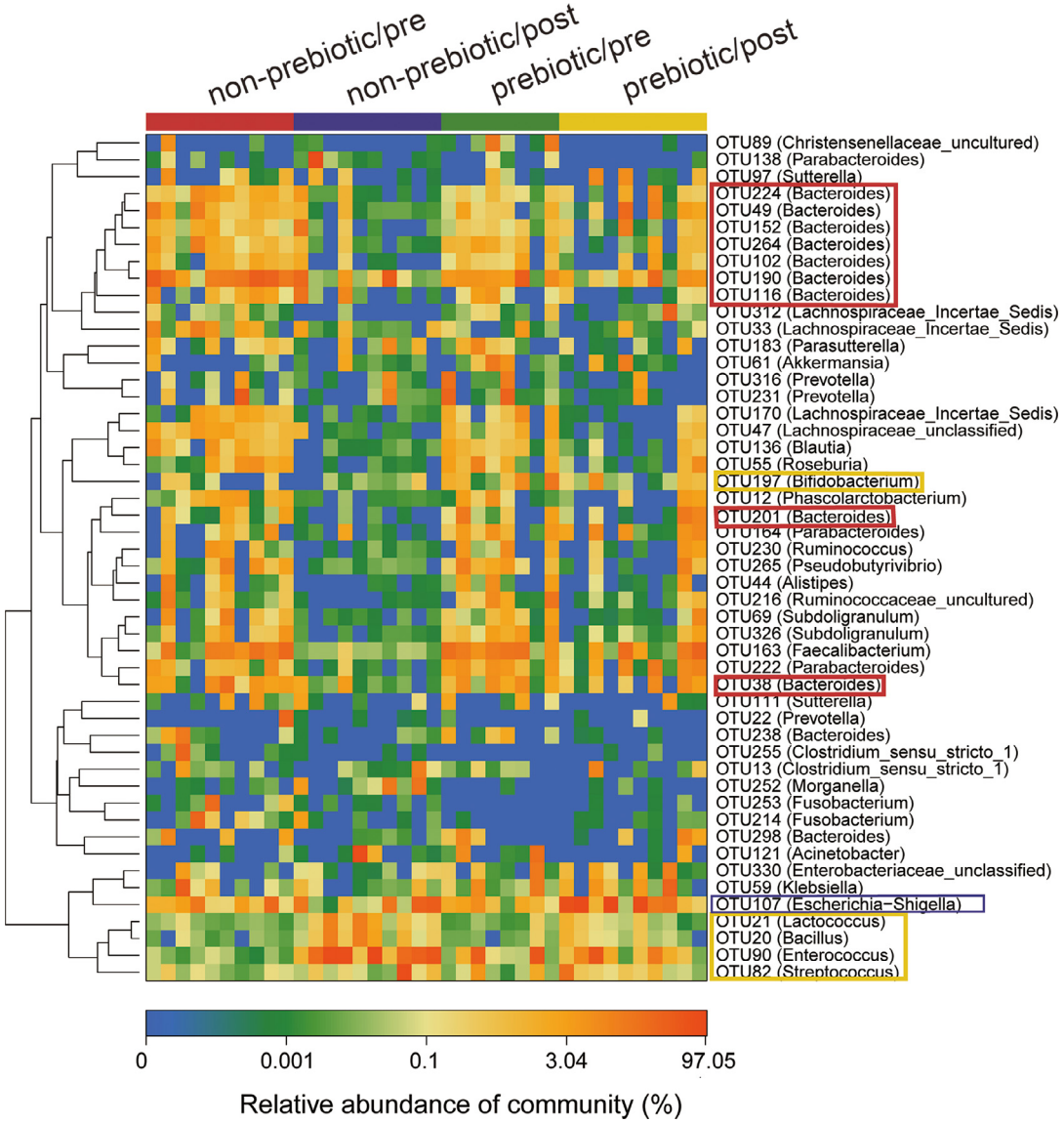

Fig. 3. Dominant microbial species at the Operational Taxonomic Unit (OUT) level. The color band was changed from blue to red, indicating that the abundance of bacteria gradually increased. Target bacteria are shown in the colored boxes. Heatmap analysis of differential intestinal microbiotas was performed using the OTU approach by using a random forest model. Microbial species were clustered according to the abundance in each sample.

does not cause human diseases [38]. Prebiotics are beneficial for the proliferation of commensals in humans. These findings indicate that prebiotics increased the abundance of commensals, but not all commensals were affected. The findings also hold the possibility of increased abundance of intestinal opportunistic pathogens. The decrease in the prevalence of *Bacteroides* highlights the presence and importance of a balanced intestinal microbiota in the human microecosystem. There maybe have a competitive relationship between some strains of *Bacteroides* and other commensals or bacteria.

The gastrointestinal tract absorbs nutrients and plays an important role in immunity because it is the central organ affected by surgical stress [39]. Surgery remains the main modality for CRC treatment [40]. Stimulation from surgical trauma and anesthesia leads to metabolic disorders and deterioration of immunologic function. Although colorectal surgery in the form of resection and anastomosis has been practiced for years, there has been no systematic assessment of the bacterial diversity and functional potential associated with surgery. In the present study we identified changes in the intestinal microbiota after tumor resection in the non-prebiotic group (Fig. 3, Table 5) by sequencing the V3–V4 region of 16S rDNA from fecal samples. Commensal microbiota (*Enterococcus, Streptococcus, Bacillus,* and *Lactococcus*) changed after operation. This phenomenon suggests that surgical stress can increase the abundance of commensal microbiota, which include opportunistic pathogenic bacteria. Surgical stress also impairs the intestinal microecological balance. It induces the transformation of opportunistic bacteria to pathogenic bacteria. Shogan et al. [41] used 16S rRNA sequencing to characterize changes in a rat model of partial colectomy and primary anastomosis. The authors found that anastomosis induced changes in the composition of the intestinal microbiota associated with the intestinal tissue, with no effect on the microbiota associated with luminal contents (i.e., stool). Most strikingly, they found a 500- and 200-fold increase in the relative abundance of *Enterococcus* and *Escherichia-Shigella*, respectively. Although the species and sampling sites of these two studies differed, both studies found increased abundance of *Enterococcus* after colon resection. Thus this

opportunistic pathogen increased as a result of surgical stress, bowel preparation, or use of antibiotics.

Escherichia is a gram-negative bacteria from the family*Enterobacteriaceae* that is commonly found in the lower intestine of warm-blooded organisms (endotherms), whereas many *Escherichia* species are commensal members of the gut microbiota [42]. *Shigella* species are human pathogens and one of the leading causes of bacterial diarrhea worldwide [43]. At the genus level, the 16S rDNA V3–V4 region of *Escherichia* and *Shigella* have the same sequence. Wang et al. [44] reported that *Escherichia-Shigella* may contribute to the differentiation of the gut microbiota of patients with CRC from that of healthy controls. We found that the abundance of *Escherichia-Shigella* was stable throughout the perioperative period, except when prebiotics were used postoperatively in the prebiotic group. One possible reason is that *Escherichia* and *Shigella* have the same sequence 16S rDNA V3–V4 region. Further, *Escherichia* is a harmless strain that is part of the normal microbiota of the gut and prevents colonization of the intestine with pathogenic bacteria through its symbiotic relationship [45]. Prebiotic supplementation may increase the abundance of harmless strains of *Escherichia*. Thus a macrogenomic approach is needed to further clarify which bacterial abundance is increased by prebiotics.

A limitation of this study was that the data collected for serum immunologic indicators and intestinal microbiota were not appreciably relevant. A different research design incorporating more patients is necessary. The technique used only identifies bacteria at the OTU level. Further research is needed to develop a more efficient method for the identification of bacteria at the species level. In addition, patients with postoperative infection complications, such as anastomotic leakage, were intentionally excluded. Further study is needed to more accurately assess the importance of microbiota changes in patients with anastomotic leakage and other complications. Finally, the microbiota was evaluated in the feces rather than in the intestine through biopsy.

Conclusions

In conclusion, prebiotic intake is recommended to improve serum immunologic indicators in patients with CRC 7 d before operation. Moreover, prebiotic supplementation changed the abundance of four commensal microbiota (*Bacteroides, Bifidobacterium, Escherichia-Shigella* and *Enterococcus*), and *Escherichia-Shigella, Bacteroides,* and *Enterococcus* are the genera known to include pathogenic strains. Stimulation from surgical trauma can alter the gut microflora, and the abundance of most intestinal microbiota was decreased postoperatively, whereas the abundance of some opportunistic pathogens and commensal microorganisms increased. *Bacteroides* is a relevant bacteria for further research on the mechanism with prebiotics. Another study is needed to address the relationship between intestinal microbiota dysbiosis and CRC to provide a basis for studying the changes in bacterial microbiota during tumor resection.

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