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ORIGINAL ARTICLE

Changes of the Intestinal Microbiota, Short Chain Fatty Acids, and Fecal pH in Patients with Colorectal Cancer

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Abstract
Background New molecular biology-based methods of bacterial identification are expected to help elucidate the relationship between colorectal cancer (CRC) and intestinal microbiota. Although there is increasing evidence revealing the potential role of microbiota in CRC, it remains unclear whether microbial dysbiosis is the cause or the result of CRC onset.
Aim We investigated the changes of intestinal environments in CRC or adenoma.
Methods We analyzed 13 groups of microbiota, 8 types of organic acids, and pH in feces obtained from the following 3 groups: individuals with CRC, adenoma, and non-adenoma. Ninety-three patients with CRC and 49 healthy individuals (22 with adenoma and 27 without adenoma) were enrolled.
Results The counts of total bacteria (10.3 ± 0.7 vs. 10.8 ± 0.3 $\log_{10}$ cells/g of feces; $p < 0.001$), 5 groups of obligate anaerobe, and 2 groups of facultative anaerobes were significantly lower in the CRC group than in the healthy individuals. While the concentrations of short chain fatty acids (SCFAs) were significantly decreased in the CRC group, the pH was increased in the CRC group (7.4 ± 0.8 vs. 6.9 ± 0.6; $p < 0.001$). Comparison among the CRC, adenoma, and non-adenoma groups revealed that fecal SCFAs and pH in the adenoma group were intermediate to the CRC group and the non-adenoma group. Within the CRC group, no differences in microbiota or organic acids were observed among Dukes stages.
Conclusions CRC patients showed significant differences in the intestinal environment, including alterations of microbiota, decreased SCFAs, and elevated pH. These changes are not a result of CRC progression but are involved in CRC onset.

Keywords Colorectal cancer · Adenoma · Microbiota · Short chain fatty acid · Fecal pH

Introduction

The human intestinal tract harbors as many as 10^{14} microorganisms [1, 2]. They play numerous beneficial

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roles, including maturation of the immune system, protection against pathogens, digestion of complex polysaccharides, and degradation of toxic substances [3, 4]. On the other hand, changes in microbial community composition are closely associated with various diseases, such as allergic disease [5], obesity [6], and intestinal inflammatory disease [7]. The association between colorectal cancer (CRC) and intestinal microbiota has been studied for many years. However, because the majority of microbiota consists of obligate anaerobes, there has been a limit to the analyses performed using conventional culture methods. Around the year 2000, molecular biological methods targeting the 16S rRNA gene brought about tremendous progress in the analysis of microbiota [8–10]. Since then, the relationship between CRC and fecal microbiota has been examined, and new data revealing the potential role of microbiota in CRC have been generated using newly developed techniques [11–13]. However, there is one question that has yet to be fully elucidated: are the changes in microbiota in CRC patients a result or a cause of the initiation of CRC? In other words, do the changes in microbiota occur during CRC progression, or do these changes result in the initiation of CRC?

Here, we examined fecal microbiota from patients with CRC at various stages and with adenoma by using reverse transcription–quantitative polymerase chain reaction (RT-qPCR), one of the newly developed techniques [14, 15]. To investigate multiple factors that could influence the intestinal environment, we focused on the organic acids fermented and generated from microbiota [16, 17] and on pH, which is closely related to the concentrations of such organic acids. Given that the intestinal environmental changes in CRC patients, we analyzed similarities and differences in the intestinal environments of 3 groups—the CRC group, the adenoma group, and the non-adenoma group—to determine whether the changes differed with the stage of CRC or adenoma.

Patients and Methods

CRC Group

A total of 101 patients who were diagnosed with primary CRC between May 2009 and October 2010 were consecutively enrolled in the CRC group. Patients with a history of colectomy or proctectomy, those with obstructive CRC, and those treated with antibiotics at the point of hospitalization were excluded. Feces were taken from the patients before starting pre-operative preparation with bowel cleansing and with oral antibiotics. Patients from whom fecal samples had not been obtained prior to these bowel preparations were excluded.

Control Group

The control group consisted of 50 healthy individuals hospitalized for medical check-ups at our hospital during the abovementioned period: 22 annually, 18 once every 2 years, and 10 once every 3 years or longer. As with the CRC group, subjects with a history of colectomy or proctectomy and those treated with antibiotics at the point of hospitalization were excluded. Subjects from whom fecal samples had not been obtained by the time laxative pretreatment for colonoscopy was started were excluded. Total colonoscopy was performed on all subjects; when a polyp was recognized, it was excised and subjected to pathological examination. All these subjects had received total colonoscopies regularly at intervals of 1–3 years; individuals with even one incidence of confirmed adenoma, identified by their records from the previous 3 years (November 2007–October 2010), were allocated to the adenoma group.

The study was approved by the hospital's review board in fiscal year 2008, and all subjects provided written informed consent for the collection of samples and subsequent analysis.

Fecal Sampling for Analysis of Microbiota, Organic Acids, and pH

Feces were collected from all hospitalized subjects. Freshly excreted feces were taken from the subjects before the start of pre-operative or pre-endoscopic preparation. Feces were placed into 2 tubes (approximately 1.0 g/tube); one tube contained 2 ml of RNA*later* (an RNA stabilization solution; Ambion, Austin, TX, USA), and the other was empty. The samples were placed in a refrigerator at 4 °C (for analysis of fecal microbiota) or in a freezer at −20 °C (for analysis of fecal organic acid concentration and fecal pH) within 30 min of excretion.

Determination of Bacterial Count by RT-qPCR

Immediately after collection, the fecal samples were weighed and then suspended in 9 volumes of RNA*later*. The fecal homogenate (200 µl) was added to 1 ml sterilized phosphate-buffered saline and then centrifuged at 5,000*g* for 10 min. Total RNA fractions were extracted from fecal pellets by the method previously described [14]. RT-qPCR analyses were performed in 384-well optical plates on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA). RT-qPCR was conducted in a one-step reaction using a OneStep RT-PCR Kit (Qiagen, Hilden, Germany) for detection and enumeration [15] of the following bacterial targets: *Clostridium coccoides* group, *C. leptum* subgroup,

Bacteroides fragilis group, *Bifidobacterium* spp., *Atopobium* cluster, and *Prevotella* spp., which are dominant intestinal bacterial groups in healthy adults. Total *Lactobacillus* spp. (*L. gasseri* subgroup, *L. brevis*, *L. casei* subgroup, *L. fermentum*, *L. fructivorans*, *L. plantarum* subgroup, *L. reuteri* subgroup, *L. ruminis* subgroup,

Table 1 16S or 23S rRNA gene-targeted primers used in this study

Target bacteria[a]	Primer	Sequence (5′–3′)	Reference
Clostridium coccoides group	g-Ccoc-F	AAATGACGGTACCTGACTAA	8
	g-Ccoc-R	CTTTGAGTTTCATTCTTGCGAA	
Clostridium leptum subgroup	sg-Clept-F	GCACAAGCAGTGGAGT	8
	sg-Clept-R3	CTTCCTCCGTTTTGTCAA	
Bacteroides fragilis group	g-Bfra-F2	AYAGCCTTTCGAAAGRAAGAT	35
	g-Bfra-R	CCAGTATCAACTGCAATTTTA	
Bifidobacterium	g-Bifid-F	CTCCTGGAAACGGGTGG	36
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA	
Atopobium cluster	g-Atopo-F	GGGTTGAGAGACCGACC	8
	g-Atopo-R	CGGRGCTTCTTCTGCAGG	
Prevotella	g-Prevo-F	CACRGTAAACGATGGATGCC	8
	g-Prevo-R	GGTCGGGTTGCAGACC	
Clostridium difficile	Cd-lsu-F	GGGAGCTTCCCATACGGGTTG	37
	Cd-lsu-R	TTGACTGCCTCAATGCTTGGGC	
Clostridium perfringens	s-Clper-F	GGGGGTTTCAACACCTCC	15
	ClPER-R	GCAAGGGATGTCAAGTGT	38
Lactobacillus casei subgroup	sg-Lcas-F	ACCGCATGGTTCTTGGC	15
	sg-Lcas-R	CCGACAACAGTTACTCTGCC	
Lactobacillus gasseri subgroup	sg-Lgas-F	GATGCATAGCCGAGTTGAGAGACTGAT	15
	sg-Lgas-R	TAAAGGCCAGTTACTACCTCTATCC	
Lactobacillus plantarum subgroup	sg-Lpla-F	CTCTGGTATTGATTGGTGCTTGCAT	15
	sg-Lpla-R	GTTCGCCACTCACTCAAATGTAAA	
Lactobacillus reuteri subgroup	sg-Lreu-F	GAACGCAYTGGCCCAA	15
	sg-Lreu-R	TCCATTGTGGCCGATCAGT	
Lactobacillus ruminis subgroup	sg-Lrum-F	CACCGAATGCTTGCAYTCACC	15
	sg-Lrum-R	GCCGCGGGTCCATCCAAAA	
Lactobacillus sakei subgroup	sg-Lsak-F	CATAAAACCTAMCACCGCATGG	15
	sg-Lsak-R	TCAGTTACTATCAGATACRTTCTTCTC	
Lactobacillus brevis	s-Lbre-F	ATTTTGTTTGAAAGGTGGCTTCGG	15
	s-Lbre-R	ACCCTTGAACAGTTACTCTCAAAGG	
Lactobacillus fermentum	LFer-1	CCTGATTGATTTTGGTCGCCAAC	15
	LFer-2	ACGTATGAACAGTTACTCTCATACGT	
Lactobacillus fructiborans	s-Lfru-F	TGCGCCTAATGATAGTTGA	15
	s-Lfru-R	GATACCGTCGCGACGTGAG	
Enterobacteriaceae	En-lsu-3F	TGCCGTAACTTCGGGAGAAGGCA	14
	En-lsu-3'R	TCAAGGACCAGTGTTCAGTGTC	
Enterococcus	g-Encoc-F	ATCAGAGGGGGATAACACTT	15
	g-Encoc-R	ACTCTCATCCTTGTTCTTCTC	
Staphylococcus	g-Staph-F	TTTGGGCTACACACGTGCTACAATGGACAA	15
	g-Staph-R	AACAACTTTATGGGATTTGCWTGA	
Pseudomonas	PSD7F	CAAAACTACTGAGCTAGAGTACG	14
	PSD7R	TAAGATCTCAAGGATCCCAACGGCT	

[a] Specific primer sets were developed by using 16S rDNA sequences except for Cd-lsu-F/R and En-lsu-3F/3'R targeting 23S rDNA

L. sakei subgroup), *Enterobacteriaceae*, *Enterococcus* spp., and *Staphylococcus* spp., which are subdominant populations but important intestinal genera for human health. *C. difficile*, *C. perfringens*, and *Pseudomonas* spp., which are opportunistic infectious pathogens. The sequences of the primers are listed in Table 1. For identification of the these bacterial population in the fecal samples, the extracted RNA from the feces was subjected to RT-qPCR, and the threshold cycle values in the linear range of the assay were applied to the analytical curve generated in the same experiment to obtain the corresponding bacterial count in each nucleic acid sample, which was converted to the count per sample.

Measurements of Fecal Organic Acid Concentrations and pH

A portion of the homogenized stool was isolated, weighed, mixed with 0.15 M perchloric acid in a fourfold volume, and allowed to stand at 4 °C for 12 h. The mixture was centrifuged at 4 °C at 20,400*g* for 10 min, and the supernatant was filtrated with a 0.45-μm membrane filter (Millipore Japan, Tokyo), and sterilized. The concentrations of organic acids were measured by using a Waters high-performance liquid chromatography system (Waters 432 Conductive Detector; Waters, Milford, MA, USA) and a Shodex Rspack KC-811 column (Showa Denko, Tokyo, Japan) [34]. We prepared a standard mixed solution consisting of 1–20 mM succinic acid, formic acid, lactic acid, acetic acid, propionic acid, butyric acid, valeric acid, and isovaleric acid and calculated the concentrations of organic acids on the basis of the standard curve. Fecal pH was measured by directly inserting the glass electrode of a D-51 pH meter (Horiba Seisakusho, Tokyo, Japan) into the homogenized feces. Measurements were taken at 3 random locations in the sample, and the mean was calculated.

Statistical Analysis

All analyses were conducted using SPSS 19.0 statistical software (IBM, Tokyo, Japan). Responses were analyzed by using descriptive statistics, including mean, variance, standard deviation (SD), and percentages. The Chi square or Fisher's exact test were used for cross-tabulated data, and the Mann–Whitney test was used to compare means of continuous data. For comparing means of continuous data among multiple groups, we used analysis of variance (ANOVA). The Bonferroni method was used for additional data analysis of the results obtained from the ANOVA. p values of less than 0.05 were considered to be statistically significant.

Results

Eligible Subjects

Out of 101 patients in the CRC group, 93 patients were eligible for the study. Eight subjects were excluded: 4 due to a past history of colectomy, 3 due to a final pathological diagnosis of tumors other than CRC (1 gastric cancer and 2 adenoma), and 1 due to insufficient quantity of stool sample. In the control group, 1 out of 50 subjects was excluded because of insufficient quantity of stool sample. Among the 49 eligible subjects, 22 were diagnosed with adenoma in the past 3 years, and 27 did not have

Table 2 Background of each group

	CRC[a] group ($n = 93$)	Healthy control group[b]		p value
		Adenoma ($n = 22$)	Non-adenoma ($n = 27$)	
Age	68.9 ± 12.1	66.6 ± 9.2	65.6 ± 13.5	0.386
Gender (male/female)	49/44	11/11	16/11	0.801
Body mass index	22.1 ± 3.3	23.4 ± 3.1	23.1 ± 3.1	0.146
P/O[c] cholecystectomy	7/93	1/22	2/27	1.000
P/O[c] appendectomy	13/93	2/22	2/27	0.690
P/O[c] breast cancer	4/93	0/22	2/27	0.582
Primary cancer site	C[d]: 5			
	A[d]: 21			
	T[d]: 9			
	D[d]: 3			
	S[d]: 20			
	R[d]: 35			

[a] *CRC* colorectal cancer

[b] Healthy control group includes the adenoma group and the non-adenoma group

[c] *P/O* post-operation

[d] *C* cecum, *A* ascending colon, *T* transverse colon, *D* descending colon, *S* sigmoid colon, *R* rectum

adenomas. The 3 groups did not differ significantly in age, gender, body mass index, or in past histories of cholecystectomy, appendectomy, or breast cancer (Table 2).

Comparison of Intestinal Environments of CRC Patients and Healthy Subjects

First, the intestinal environment was compared between 2 groups: the CRC group and the healthy control group (consisting of the adenoma group and the non-adenoma group) (Table 3). Total bacterial counts in the CRC group were significantly lower than those in the healthy group (10.3 ± 0.7 vs. 10.8 ± 0.3 $\log_{10}$ cells/g of feces; $p < 0.001$). Specifically, the counts of 5 groups of obligate anaerobes (*C. coccoides* group, *C. leptum* subgroup, *Bacteroides fragilis* group, *Bifidobacterium*, and *Atopobium* cluster) and 2 groups of facultative anaerobes (*Enterobacteriaceae* and *Staphylococcus*) were significantly lower in the CRC group. In contrast, the bacterial counts of *C. difficile*, *C. perfringens*, and *Pseudomonas*—highly pathogenic species—were greater in the CRC group, although this difference was not significant.

Total organic acids concentrations were significantly lower in the CRC group than in the healthy group (75.9 ± 39.5 vs. 99.1 ± 30.0 μmol/g of feces; $p < 0.001$). Specifically, the concentrations of acetic acid, propionic acid, butyric acid, and valeric acid—classified as short chain fatty acid (SCFA)—were decreased. Conversely, the concentration of succinic acid was significantly increased in the CRC group (3.1 ± 4.7 vs. 2.8 ± 9.7; $p < 0.05$). Fecal pH in the CRC group was significantly higher than in the healthy group (7.4 ± 0.8 vs. 6.9 ± 0.6; $p < 0.001$).

Table 3 Comparison of intestinal environments of CRC patients and healthy individuals

	CRC group ($n = 93$)	Healthy control group ($n = 49$)	p value
Total bacterial counts	**10.3 ± 0.7 (93/93)**	**10.8 ± 0.3 (49/49)**	**<0.001**
Obligate anaerobe			
***Clostridium coccoides* group**	**9.5 ± 0.7 (93/93)**	**9.9 ± 0.4 (49/49)**	**0.004**
***C. leptum* subgroup**	**9.4 ± 0.8 (93/93)**	**10.1 ± 0.4 (49/49)**	**<0.001**
***Bacteroides fragilis* group**	**9.6 ± 0.8 (91/93)**	**10.2 ± 0.4 (49/49)**	**<0.001**
Bifidobacterium	**9.3 ± 0.9 (91/93)**	**9.7 ± 0.6 (49/49)**	**0.006**
***Atopobium* cluster**	**9.0 ± 0.7 (91/93)**	**9.5 ± 0.5 (49/49)**	**<0.001**
Prevotella	8.2 ± 1.4 (58/93)	8.1 ± 1.8 (25/49)	0.897
C. difficile	5.0 ± 1.1 (10/93)	5.0 ± 0.9 (2/49)	0.919
C. perfringens	5.5 ± 1.7 (53/93)	5.1 ± 1.3 (27/49)	0.448
Facultative anaerobe			
Total *Lactobacillus*	6.9 ± 1.6 (93/93)	7.0 ± 1.3 (49/49)	0.695
Enterobacteriaceae	**7.2 ± 1.0 (78/93)**	**7.6 ± 1.1 (49/49)**	**0.026**
Enterococcus	6.6 ± 1.4 (80/93)	6.8 ± 1.4 (49/49)	0.666
Staphylococcus	**4.3 ± 0.7 (76/93)**	**4.8 ± 0.7 (49/49)**	**<0.001**
Aerobe			
Pseudomonas	4.9 ± 1.1 (19/93)	4.3 ± 1.0 (6/49)	0.127
Total organic acids	**75.9 ± 39.5 (93/93)**	**99.1 ± 30.0 (49/49)**	**<0.001**
Succinic acid	*3.1 ± 4.7 (64/93)*	*2.8 ± 9.7 (30/49)*	*0.020*
Formic acid	1.8 ± 2.2 (70/93)	1.7 ± 1.0 (40/49)	0.170
Lactic acid	3.0 ± 3.4 (29/93)	3.2 ± 4.5 (10/49)	0.676
Acetic acid	**49.3 ± 27.2 (93/93)**	**59.6 ± 19.2 (49/49)**	**0.002**
Propionic acid	**12.7 ± 8.1 (91/93)**	**19.8 ± 6.6 (49/49)**	**<0.001**
Butyric acid	**7.7 ± 4.7 (88/93)**	**11.8 ± 4.9 (49/49)**	**<0.001**
Valeric acid	**2.8 ± 1.2 (37/93)**	**3.9 ± 1.7 (23/49)**	**0.029**
Isovaleric acid	2.8 ± 1.9 (44/93)	3.6 ± 1.9 (31/49)	0.063
pH	*7.4 ± 0.8 (93/93)*	*6.9 ± 0.6 (49/49)*	*<0.001*

Bacterial counts are expressed as the mean ± SD ($\log_{10}$ bacterial cells/g of feces). *SD* standard deviation

Organic acid concentrations are expressed as the mean ± SD (μmol/g of feces)

Parentheses indicate: the number of patients in whom microbiota, organic acids, and pH was detected divided by the number of patients tested

The items highlighted in bold are those that are significantly lower in the CRC group than in the healthy control group (Mann–Whitney test)

The items highlighted in italic are those that are significantly higher in the CRC group than in the healthy control group (Mann–Whitney test)

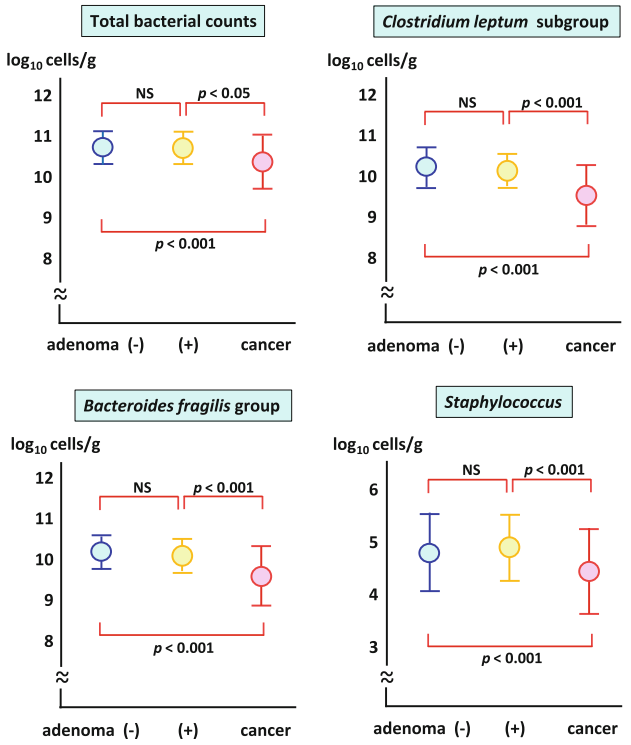

Fig. 1 Comparison of microbiota among the CRC group, adenoma group, and non-adenoma group. Bacterial counts are expressed as the mean ± SD ($\log_{10}$ bacterial cells/g of feces). *SD* standard deviation. Significant differences were observed between the CRC group and the non-adenoma group as well as between the CRC group and the adenoma group (Bonferroni method)

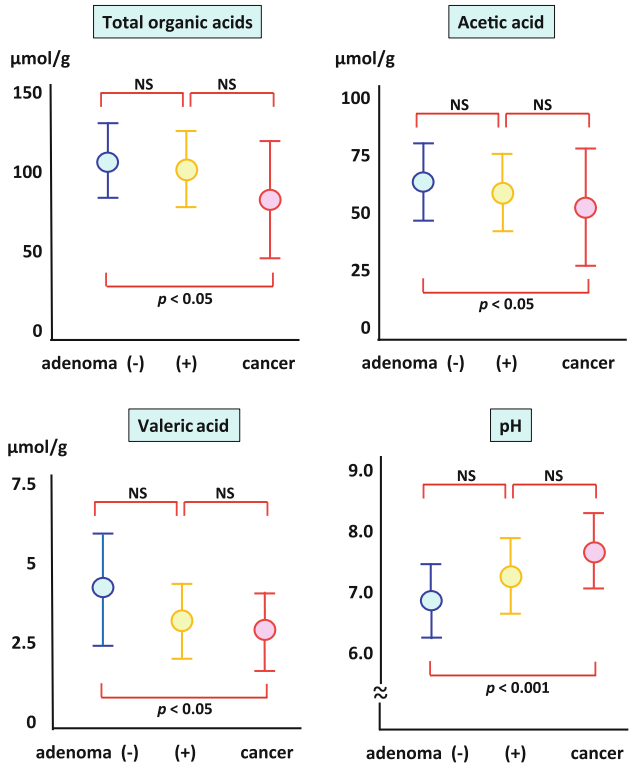

Fig. 2 Comparison of organic acids and pH among the CRC group, adenoma group, and non-adenoma group. Organic acid concentrations (μmol/g of feces) and pH are expressed as the mean ± SD. Significant differences were observed between the CRC group and the non-adenoma group, while no difference was observed between the CRC group and the adenoma group (Bonferroni method); the concentrations of total organic acids, acetic acid, valeric acid, and fecal pH in the adenoma group were intermediate to the CRC group and the non-adenoma group

Comparison of Intestinal Environments of the CRC Group, Adenoma Group, and Non-adenoma Group

We compared the intestinal environments of 3 groups: the CRC group ($n = 93$), adenoma group ($n = 22$), and non-adenoma group ($n = 27$) using ANOVA. Firstly, significant differences were observed in total bacterial counts ($p < 0.001$) and the counts of 6 bacterial groups (*C. coccoides* group, $p < 0.005$; *C. leptum subgroup*, $p < 0.001$; *Bacteroides fragilis* group, $p < 0.001$; *Bifidbacterium*, $p = 0.013$; *Atopobium* cluster, $p < 0.001$; and *Staphylococcus*, $p = 0.001$). Among the 3 groups, significant differences were observed between the CRC group and the non-adenoma group as well as between the CRC group and the adenoma group using Bonferroni method (Fig. 1).

Secondly, concerning organic acids of the 3 groups, significant differences were observed in the concentration of total organic acids ($p < 0.001$), acetic acid ($p = 0.037$), propionic acid ($p < 0.001$), btyric acid ($p < 0.001$), and valeric acid ($p = 0.006$). This comparison of organic acid concentrations among the 3 groups, however, revealed characteristics different from those observed in the microbiota analyses. The CRC group had lower total organic acids, acetic acid, and valeric acid concentrations than the non-adenoma group, while no difference was observed between the CRC group and the adenoma group (Fig. 2). The concentrations of total organic acids, acetic acid, and valeric acid in the adenoma group were approximately half those of the CRC group and the non-adenoma group.

Thirdly, fecal pH revealed a significant difference especially between the CRC group and the non-adenoma group (7.4 ± 0.8 vs. 6.8 ± 0.6; $p < 0.001$), while the adenoma group's pH was intermediate to the CRC group and the non-adenoma group using Bonferroni method (Fig. 2).

Comparison of Intestinal Environments According to CRC Stage

Within the CRC group, ANOVA was performed among four Dukes stages: Dukes A (36 patients), Dukes B (19 patients), Dukes C (24 patients), and Dukes D (14 patients) (Table 4). There were no significant differences in microbiota or organic acids among stages. Only fecal pH revealed significant difference among the four stages using

Table 4 Comparison of intestinal environments according to Dukes stage in the CRC group

	Dukes A ($n = 36$)	Dukes B ($n = 19$)	Dukes C ($n = 24$)	Dukes D (n = 14)	p value
Total bacterial counts	10.3 ± 0.7 (36/36)	10.3 ± 0.7 (19/19)	10.4 ± 0.6 (24/24)	10.0 ± 0.8 (14/14)	0.502
Obligate anaerobe					
Clostridium coccoides group	9.6 ± 0.7 (36/36)	9.5 ± 0.9 (19/19)	9.6 ± 0.6 (24/24)	9.1 ± 0.8 (14/14)	0.265
C. leptum subgroup	9.4 ± 0.8 (36/36)	9.3 ± 0.7 (19/19)	9.6 ± 0.6 (24/24)	9.0 ± 0.9 (14/14)	0.070
Bacteroides fragilis group	9.7 ± 0.7 (34/36)	9.4 ± 0.9 (19/19)	9.6 ± 0.8 (24/24)	9.5 ± 0.8 (14/14)	0.592
Bifidobacterium	9.4 ± 0.8 (34/36)	9.4 ± 1.1 (19/19)	9.2 ± 1.0 (24/24)	9.0 ± 1.1 (14/14)	0.487
Atopobium cluster	9.0 ± 0.6 (35/36)	9.1 ± 0.9 (19/19)	8.9 ± 0.6 (24/24)	8.6 ± 0.6 (13/14)	0.265
Prevotella	8.6 ± 1.5 (21/36)	8.6 ± 1.1 (12/19)	7.9 ± 1.4 (17/24)	7.2 ± 1.3 (8/14)	0.068
C. difficile	4.1 ± 0.9 (3/36)	4.4 ± 1.3 (2/19)	4.1 ± 0.8 (2/24)	4.4 ± 0.4 (3/14)	0.400
C. perfringens	5.5 ± 2.3 (19/36)	6.0 ± 1.5 (9/19)	5.5 ± 2.1 (17/24)	5.2 ± 1.4 (8/14)	0.736
Facultative anaerobe					
Total Lactobacillus	6.8 ± 1.6 (36/36)	7.2 ± 1.4 (19/19)	7.0 ± 1.5 (24/24)	6.6 ± 1.9 (14/14)	0.691
Enterobacteriaceae	7.3 ± 1.1 (30/36)	7.2 ± 1.1 (17/19)	7.1 ± 0.9 (20/24)	6.9 ± 0.6 (11/14)	0.645
Enterococcus	6.7 ± 1.8 (30/36)	6.5 ± 1.3 (15/19)	6.7 ± 1.2 (22/24)	6.7 ± 1.2 (13/14)	0.989
Staphylococcus	4.2 ± 0.7 (30/36)	4.4 ± 0.8 (16/19)	4.4 ± 0.6 (20/24)	5.3 ± 1.2 (10/14)	0.798
Aerobe					
Pseudomonas	4.8 ± 1.3 (6/36)	5.1 ± 1.4 (3/19)	4.5 ± 1.0 (5/24)	5.3 ± 1.2 (5/14)	0.793
Total organic acids	67.1 ± 33.4 (36/36)	85.9 ± 40.0 (19/19)	87.3 ± 48.4 (24/24)	65.1 ± 30.7 (14/14)	0.109
Succinic acid	3.4 ± 6.0 (21/36)	4.3 ± 5.4 (15/19)	2.0 ± 2.0 (18/24)	2.8 ± 4.4 (10/14)	0.565
Formic acid	1.2 ± 0.9 (25/36)	2.0 ± 1.7 (13/19)	1.8 ± 1.5 (21/24)	2.8 ± 4.6 (11/14)	0.224
Lactic acid	1.7 ± 2.4 (12/36)	3.8 ± 3.7 (7/19)	4.7 ± 6.1 (4/24)	3.5 ± 2.3 (6/14)	0.336
Acetic acid	44.1 ± 22.4 (36/36)	55.4 ± 29.3 (19/19)	56.1 ± 34.2 (24/24)	42.9 ± 19.5 (14/14)	0.212
Propionic acid	11.6 ± 7.4 (34/36)	13.5 ± 7.1 (19/19)1	5.0 ± 10.5 (24/24)	10.2 ± 5.5 (14/14)	0.257
Butyric acid	7.3 ± 4.1 (34/36)	7.7 ± 4.5 (19/19)	9.6 ± 5.6 (22/24)	5.3 ± 3.8 (13/14)	0.067
Valeric acid	2.8 ± 1.2 (11/36)	2.5 ± 1.6 (11/19)	2.9 ± 1.1 (13/24) :	3.1 ± 10.5 (2/14)	0.852
Isovaleric acid	2.6 ± 1.8 (13/36)	2.5 ± 1.7 (11/19)	3.3 ± 1.9 (14/24)	2.3 ± 2.6 (5/14)	0.662
pH	**7.4 ± 0.7 (36/36)**	**7.3 ± 0.8 (19/19)**	**7.3 ± 0.7 (24/24)**	**8.0 ± 0.7 (14/14)**	0.043

The item highlighted in *bold* shows a significant difference (ANOVA)

Although a significant difference is observed in fecal pH, there is no significant difference among the respective Dukes stages (Bonferroni method)

ANOVA ($p = 0.043$), and pH of Dukes D was the highest (pH = 8.0 ± 0.7). However, there was no significant difference in fecal pH among the respective Dukes stages using Bonferroni method.

Comparison of Intestinal Environments According to Tumor Sites in the CRC Group

Ninety-three CRC patients were divided into 3 groups according to tumor sites for comparison of intestinal environments: a group of 31 patients with colon cancer located between the cecum and the middle of the transverse colon (right side), a group of 27 with colon cancer located between the left side of the transverse colon and the sigmoid colon (left side), and a group of 35 with rectal cancer (rectum). Except for counts of *C. perfringens* in microbiota ($p = 0.007$), there were no significant differences in organic acids concentrations or fecal pH according to tumor sites using ANOVA. The count of *C. perfringens* was significantly decreased in the left side group as compared with the right side ($p = 0.009$) and rectum ($p = 0.038$) groups (Table 5).

Discussion

The CRC group showed significant changes in fecal microbiota, significant decreases in fecal SCFAs concentrations, and a significant increase in fecal pH compared with the healthy individuals (adenoma or non-adenoma); however, no differences in these factors were observed among Dukes stages except for pH. Total organic acids concentrations, SCFAs (particularly acetic acid and valeric acid) concentrations, and pH values in the adenoma group were approximately half those of the CRC group and the non-adenoma group. Taken together, our findings strongly

Table 5 Comparison of intestinal environments according to tumor sites in the CRC group

	Right side ($n = 31$)	Left side ($n = 27$)	Rectum ($n = 35$)	p value
Total bacterial counts	10.3 ± 0.7 (31/31)	10.1 ± 0.7 (27/27)	10.4 ± 0.7 (35/35)	0.400
Obligate anaerobe				
Clostridium coccoides group	9.5 ± 0.7 (31/31)	9.3 ± 0.8 (27/27)	9.6 ± 0.7 (35/35)	0.469
C. leptum subgroup	9.5 ± 0.6 (31/31)	9.2 ± 0.9 (27/27)	9.4 ± 0.8 (35/35)	0.462
Bacteroidesfragilis group	9.4 ± 0.8 (30/31)	9.6 ± 0.8 (27/27)	9.7 ± 0.7 (34/35)	0.262
Bifidobacterium	9.1 ± 0.9 (31/31)	9.2 ± 1.0 (26/27)	9.5 ± 0.9 (34/35)	0.230
Atopobium cluster	9.0 ± 0.7 (31/31)	8.8 ± 0.7 (26/27)	9.1 ± 0.6 (34/35)	0.118
Prevotella	8.2 ± 1.7 (23/31)	7.7 ± 1.2 (12/27)	8.4 ± 1.3 (23/35)	0.427
C. difficile	4.6 ± 0.5 (4/31)	3.8 (1/27)	5.1 ± 1.4 (5/35)	0.542
C. perfringens	**6.2 ± 1.6 (17/31)**[#]	**4.5 ± 1.3 (17/27)**[#¶]	**5.9 ± 1.8 (17/35)**[¶]	0.007
Facultative anaerobe				
Total *Lactobacillus*	7.3 ± 1.9 (31/31)	6.8 ± 1.3 (27/27)	6.8 ± 1.5 (35/35)	0.360
Enterobacteriaceae	7.1 ± 1.1 (26/31)	7.4 ± 1.0 (23/27)	7.1 ± 0.9 (29/35)	0.423
Enterococcus	7.0 ± 1.6 (26/31)	6.7 ± 1.5 (23/27)	6.3 ± 1.2 (31/35)	0.212
Staphylococcus	4.3 ± 0.7 (26/31)	4.2 ± 0.7 (20/27)	4.4 ± 0.7 (30/35)	0.392
Aerobe				
Pseudomonas	4.7 ± 1.5 (4/31)	4.4 ± 1.1 (5/27)	5.2 ± 1.0 (10/35)	0.383
Total organic acids	68.3 ± 36.6 (31/31)	76.2 ± 35.1 (27/27)	82.3 ± 44.7 (35/35)	0.363
Succinic acid	2.1 ± 2.2 (22/31)	2.4 ± 3.6 (19/27)	4.7 ± 6.7 (23/35)	0.122
Formic acid	1.8 ± 1.4 (23/31)	1.3 ± 0.9 (19/27)	2.2 ± 3.2 (28/35)	0.417
Lactic acid	2.7 ± 3.8 (12/31)	2.2 ± 2.4 (9/27)	4.3 ± 3.8 (8/35)	0.423
Acetic acid	44.6 ± 23.0 (31/31)	48.9 ± 23.1 (27/27)	53.9 ± 33.0 (35/35)	0.381
Propionic acid	1.7 ± 8.2 (30/31)	14.3 ± 9.3 (27/27)	12.3 ± 7.1 (34/35)	0.438
Butyric acid	7.0 ± 4.2 (29/31)	7.6 ± 4.7 (27/27)	8.3 ± 5.2 (32/35)	0.529
Valeric acid	2.7 ± 1.7 (12/31)	2.7 ± 0.9 (9/27)	2.9 ± 1.1 (16/35)	0.914
Isovaleric acid	2.3 ± 1.8 (14/31)	3.1 ± 2.2 (10/27)	2.9 ± 1.8 (20/35)	0.581
pH	7.4 ± 0.7 (31/31)	7.5 ± 0.8 (27/27)	7.4 ± 0.8 (35/35	0.730

The item highlighted in *bold* shows a significant difference (ANOVA)

# indicates a significant difference ($p = 0.009$) between the *Right side* group and the *Left side* group (Bonferroni method)

¶ indicates a significant difference ($p = 0.038$) between the *Left side* group and the *Rectum* group (Bonferroni method)

suggest that it is not the progression of CRC that causes changes in the intestinal environment but rather that the cancer initiates and progresses in an intestinal environment that has changed. In addition, the counts of many bacterial species—particularly obligate anaerobes—were decreased in the CRC group, demonstrating that microbial community composition changes collectively in CRC. It has been previously reported that specific bacterial species, such as *Streptococcus bovis* [18, 19] or *Bacteroides* [20, 21], are involved in CRC. We cannot rule out the possibility that some bacterial species have strong effects individually; however, notably, our analyses revealed that the microbiota themselves undergo collective changes.

Our study demonstrated that, in the CRC group, the concentrations of SCFAs such as acetic acid, propionic acid, and butyric acid were markedly decreased. These 3 types of organic acids are usually the most abundant in the intestinal tract. Although organic acids concentrations are reportedly higher in the right side than the left side of the human large intestine [33], our study revealed no difference in organic acids concentrations according to CRC sites. Organic acids are carbon-based organic compounds with acidic properties. In the gastrointestinal tract, the microbiota is the most active producer of organic acids. In addition to being the main energy sources for the intestinal mucosa [22], organic acids play multiple roles, including prevention of infection by maintaining intestinal acidity, suppression of absorption of toxic substances, and promotion of cancer cell apoptosis via p21 activity [17, 23]. Specifically, Bird et al. [16] have reported that butyrate reduces the risk of CRC. In our study, changes in microbial community appeared to have affected the production of organic acids; this may have either directly or indirectly promoted carcinogenesis. Furthermore, our results demonstrated that the changes in fecal

organic acids concentrations and pH occurred not only in CRC but also in adenoma. Colorectal carcinogenesis involves both genetic [24] and environmental factors [25]. Individual genetic factors have already been identified as playing important roles in the multistep progression from normal mucosa to adenoma and finally to cancer [26]. However, environmental factors—particularly dietary factors—are also intimately involved in carcinogenesis [25]. Our results suggest that intestinal environmental changes are the key to progression toward adenoma and then to cancer.

Our results may be applicable to the prevention of CRC. We hypothesize that it may be possible to prevent CRC by improving the intestinal environment because the decrease in counts of microbiota (particularly obligate anaerobes) leads to changes in the intestinal environment, which may ultimately lead to the development of CRC. In fact, the administration of probiotics to CRC patients can alleviate postoperative complications [27, 28], and the use of prebiotics and synbiotics may boost immunity, reducing the risk of developing CRC [11, 29]. Our results may also be useful for identifying groups at high risk for CRC. Currently, 2-day fecal occult blood testing is widely performed for screening for CRC; however, this method does not have high sensitivity or specificity, making its reliability questionable [30]. By adding the parameters of intestinal environment to the screening system, groups at high risk could be further identified.

Our study has several limitations. Firstly, since it was a cross-sectional study targeting CRC patients with diverse backgrounds, the number of patients was small and even smaller when divided into each Dukes stage. Secondly, the sample sizes of the control adenoma and non-adenoma groups were small with less than 30 patients each. Finally, regional and racial differences reported in microbiota [31, 32] were not taken into account. However, despite these limitations, particularly the small sample sizes, our study demonstrated intestinal environmental factors, such as microbiota, SCFAs, and pH, to differ markedly in CRC patients from those in healthy individuals. Furthermore, these intestinal environmental factors did not change as the cancer progressed. These results indicate that the changes in intestinal environmental factors might have occurred in the early stage of carcinogenesis. Thus, our study is extremely significant because it has disclosed the possibility that CRC may have initiated in the already altered intestinal environment.

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Conflicts of interest None.

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