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Original Article

The Possible Preventative Role of Lactate- and Butyrate-Producing Bacteria in Colorectal Carcinogenesis

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Background/Aims: The gut microbiome has emerged as a key player that mechanistically links various risk factors to colorectal cancer (CRC) etiology. However, the role of the gut microbiome in CRC pathogenesis remains unclear. This study aimed to characterize the gut microbiota in healthy controls (HCs) and patients with colorectal adenoma (AD) and CRC in subgroups based on sex and age.

Methods: Study participants who visited the hospital for surveillance of CRC or gastrointestinal symptoms were prospectively enrolled, and the gut microbiome was analyzed based on fecal samples.

Results: In terms of HC-AD-CRC sequence, commensal bacteria, including lactate-producing (*Streptococcus salivarius*) and butyrate-producing (*Faecalibacterium prausnitzii*, *Anaerostipes hadrus*, and *Eubacterium hallii*) bacteria, were more abundant in the HC group than in the AD and CRC groups. In the sex comparison, the female HC group had more lactate-producing bacteria (*Bifidobacterium adolescentis*, *Bifidobacterium catenulatum*, and *Lactobacillus ruminis*) than the male HC group. In age comparison, younger subjects had more butyrate-producing bacteria (*Agathobaculum butyriciproducens* and *Blautia faecis*) than the older subjects in the HC group. Interestingly, lactate-producing bacteria (*B. catenulatum*) were more abundant in females than males among younger HC group subjects. However, these sex- and age-dependent differences were not observed in the AD and CRC groups.

Conclusions: The gut microbiome, specifically lactate- and butyrate-producing bacteria, which were found to be abundant in the HC group, may play a role in preventing the progression of CRC. In particular, lactate-producing bacteria, which were found to be less abundant in healthy male controls may contribute to the higher incidence of CRC in males. **(Gut Liver 2024;18:654-666)**

Key Words: Age distribution; Biomarkers; Colorectal neoplasms; Gastrointestinal microbiome; Sex

INTRODUCTION

Colorectal adenoma (AD) is considered a major precursor of colorectal cancer (CRC),[1] so-called colorectal AD-CRC sequence. It is affected by older age, a family history of cancer, smoking, and high consumption of red and processed meat.[2] The higher incidence of CRC in males than in females[3] suggests that estrogen, a female sex hormone, exerts a protective effect against CRC development.[4,5] Furthermore, estrogen reportedly modulates the composition of the gut microbiota. and conversely, estrogen levels are strongly influenced by the gut microbiome.[6] For instance, the gut microbiome was altered in ovariectomized rodents, mimicking the human postmenopausal condition.[7,8] In addition, obesity, diabetes, and cancer adversely affect the crosstalk between estrogen and the gut microbiome,[9] as

well as sex, age, and probiotics administration.[10-13]

Recently, the gut microbiome has emerged as a key player that mechanistically links various risk factors to CRC etiology. Previous metagenomic analyses have suggested that CRC is associated with gut bacterial dysbiosis.[14] Proteobacterial induction reportedly correlated with the increased expression of oncogenic genes in the azoxymethane and dextran sodium sulfate-induced CRC mouse model.[15] In contrast, dietary fiber intake enriches *Bifidobacterium* and *Lactobacillus* spp., which ferment dietary fiber into short-chain fatty acids (SCFAs).[16] SCFAs exert protective effects against CRC through various mechanisms, including regulation of the regulatory T-cell homeostasis and epigenetic transformation of tumor cells via inhibition of histone deacetylases.[17] Interestingly, SCFA levels were markedly downregulated in ovariectomized rats.[7] Furthermore, the fecal concentration of SCFAs in healthy Spanish patients decreased with age; patients >80 years of age displayed less than half the SCFA levels found in younger adults (<50 years old).[18] However, the role of the gut microbiome in CRC pathogenesis remains unclear.

We hypothesized that changes in the gut microbiota and consequent alterations in bacterial functions may contribute to the HC-colorectal AD-CRC sequence. Thus, this study aimed to characterize changes in the gut microbiota in the HC-AD-CRC sequence and changes according to sex and age.

MATERIALS AND METHODS

1. Study design and subjects

The study design is illustrated in Fig 1. Study participants who visited Seoul National University Bundang Hospital from January 2021 to December 2022 for regular check-ups for CRC surveillance or gastrointestinal symptoms were prospectively enrolled. Subjects who had no evidence of CRC or AD were included as healthy controls (HCs). Histologically confirmed CRC or colorectal AD were allocated as disease group. The following patients were excluded: (1) those with a history of CRC or colectomy before the first surveillance colonoscopy; (2) those with incomplete colonoscopy or clinical information; and (3) those who took antibiotics for the past 6 months. The following data were collected from participant questionnaires and medical records: sex, age, body mass index, and social history, such as alcohol consumption and smoking. This study was approved by the Institutional Review Board of Seoul National University Bundang Hospital (IRB number: B-1305/203-009) and was conducted in accordance with the ethical guidelines of the Declaration of Helsinki (1898). Informed consent was obtained from all participants.

2. Stool DNA extraction and sequencing

Fecal samples (≥5 g per participant) were collected and immediately frozen at –20°C at home the day before the hospital visit. They were later stored at –80°C in the laboratory until analysis. Genomic DNA was extracted using

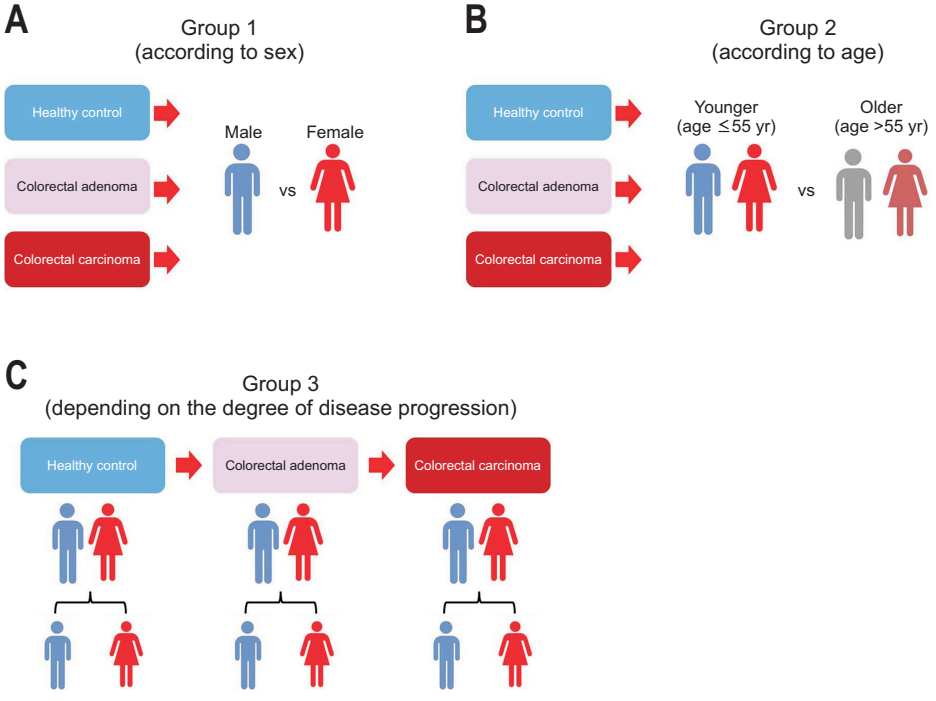


Fig. 1. Analysis scheme. Gut microbiome data were obtained from fecal samples of 56 participants, including 21 healthy controls, 21 patients with colorectal adenoma, and 14 patients with colorectal cancer. Patients were further classified into two groups based on sex (male and female) and age (≤55 and >55 years).

the QIAamp DNA Stool Mini Kit (Qiagen, Germantown MD, USA). Polymerase chain reaction and metagenome sequencing were conducted on the isolated DNA at CJ Bioscience, Inc. (Seoul, South Korea). The polymerase chain reaction utilized specific primers for the 16S rRNA V3–V4 regions (forward, 5'-AATGATACGGCGACCACCGAGATCTACAC-XXXXXXXX-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-CCTACGGGNGGCWGCAG-3' and reverse, 5'-CAAGCAGAAGACGGCATACGAGATXXXXXXXX-GTCTCGTGGGCTCGG-AGATGTGTATAAGAGACAG-GACTACHVGGGTATCTAATCC-3'). Mixed amplicons were pooled and sequencing was performed using the Illumina MiSeq sequencing system.

3. Data processing

Data processing and analysis were performed by CJ Bioscience Inc.[19] The paired-end sequencing data were merged using PANDAseq.[20] The primer sequences were then trimmed using an in-house program of the CJ Bioscience, Inc. with a similarity cutoff of 0.8. Nonspecific amplicons, which do not encode 16S rRNA, were identified using the HMMER program hmmsearch based on the 16S rRNA profiles.[21] The sequences were denoised using DUDE-Seq,[22] and the non-redundant reads were extracted using the UCLUST clustering algorithm.[23] The EzBioCloud database (CJ Bioscience, Inc.) was used for taxonomic assignment with USEARCH (8.1.1861_i86linux32).[23] Precise pairwise alignment was performed using UCHIME.[24] The non-chimeric 16S rRNA database was used to detect chimeras for reads with a best hit similarity rate of less than 97%. The sequence data were then clustered using CD-HIT[25] and UCLUST.[23]

4. Microbiome analysis

Rarefaction curves for operational taxonomic units were generated using EzBioCloud, with reads normalized to 4,612 for analysis. Alpha diversity of the gut microbiome was assessed using EzBioCloud. Differences in microbiome composition between groups were visualized through principal coordinate analysis (PCoA), and samples were clustered based on PCoA results. Beta diversity analysis, including PCoA, was conducted using the generalized UniFrac method at the species level. Significance of group separation was determined using permutational multivariate analysis of variance. A taxonomic bar graph was created to display relative operational taxonomic unit abundance (%) at the phylum level using EzBioCloud. Microbial abundances (%) at various taxonomic levels (phylum, class, order, family, genus, and species) can be found in the Supplementary Dataset.

5. Determination of taxonomic biomarkers

Taxonomic biomarkers were identified and their significance was assessed using the linear discriminant analysis effect size (LEfSe) method[26] at the species level. LEfSe data were further simplified into a Venn diagram.

6. Statistical analysis

Statistical analyses, excluding beta diversity, were conducted using PASW Statistics version 18.0.0 (SPSS Inc., Chicago, IL, USA). Beta diversity statistical data were generated using EzBioCloud. Group comparisons were initially made using the Kruskal-Wallis H test, followed by a Mann-Whitney U-test to compare the two groups, with significance considered at $p<0.05$.

7. Data availability

The datasets generated for this study can be found in the NCBI Sequence Read Archive Database (SRA accession number PRJNA940311) (https://www.ncbi.nlm.nih.gov/sra/PRJNA940311).

RESULTS

Finally, 56 participants, including 21 HCs, 21 patients with AD, and 14 patients with CRC, were enrolled (Table 1). The age of CRC was oldest and that of control was youngest ($p<0.001$). In addition, there was a sex difference among three groups ($p=0.036$). The gut microbiome data were divided into group 1 (sex), group 2 (age), and group 3 (HC-AD-CRC) according to the analysis scheme in Fig. 1.

1. Effect of sex on the gut microbiome composition

The HC, AD, and CRC samples were analyzed by sex to assess their impact on bacterial composition. Differences in taxonomic abundance at the phylum level were examined using bar graphs and scatter plots. In the HC group, Firmicutes abundance was significantly lower in females compared to males ($p=0.005$). Conversely, Bacteroidetes abundance was higher in females but not significantly different from males in HCs. The ratio of Firmicutes to Bacteroidetes (F/B) was significantly lower in females compared to males in the HC group ($p=0.021$). However, in the AD and CRC groups (Fig. 2), these sex-related differences in taxonomic abundance disappeared, and the trend in Firmicutes and Bacteroidetes abundance reversed between males and females. Moreover, there were no sex differences in beta diversity within the groups (Supplementary Fig. 1).

Sex-based differences in the gut microbiome within each group were analyzed using the LEfSe method. In the HC group, there were 18 species showing sex-based differ-

Table 1. Baseline Characteristics of the Study Cohort

Characteristic	HC (n=21)	AD (n=21)	CRC (n=14)	p-value
Age, yr	48.0±11.2	65.1±10.2	66.5±13.3	0.001†
≤55	16 (76.2)	5 (23.8)	3 (21.4)	
>55	5 (23.8)	16 (76.2)	11 (78.6)	
Sex				0.036†
Male	14 (66.7)	7 (33.3)	10 (71.4)	
Female	7 (33.3)	14 (66.7)	4 (28.6)	
Body mass index, kg/m^2	23.3±2.6	24.9±3.9	25.4±4.8	0.377
Male	23.2±2.1	26.2±3.2	26.6±4.6	
Female	23.4±3.1	24.3±3.9	26.6±3.2	
Smoking*				0.294
Non-smoker	3 (75.0)	16 (84.2)	7 (58.3)	
Current/ex-smoker	1 (25.0)	3 (15.8)	5 (41.7)	
Alcohol drinking*				0.141
No	5 (45.5)	13 (68.4)	4 (33.3)	
Yes	6 (54.5)	6 (31.6)	8 (66.7)	
Histologic type	-		-	
Tubular		15 (71.4)		
Tubulovillous		6 (28.6)		
Tumor invasion depth	-	-		
pTis			6 (42.9)	
pT1			2 (14.3)	
pT2			0	
pT3			5 (35.7)	
pT4			1 (7.1)	

Date are presented as mean±SD or number (%).
HC, healthy control; AD, colorectal adenoma; CRC, colorectal cancer.
*There were missing values. The significance of the association between variables was assessed using the chi-square test. When there was a cell with an expected frequency of less than five in categorical variables, the Fisher exact test was used instead;†Statistical significance.

ences, including 11 commensal and seven uncharacterized species. Among these, three commensal bacteria (*Eubacterium eligens*, *Clostridium nexile*, and *Alistipes shahii*) were more abundant in males, while eight commensal bacteria (*Akkermansia muciniphila*, *Bacteroides dorei*, *Bifidobacterium adolescentis*, *Bifidobacterium catenulatum*, *Alistipes onderdonkii*, *Lactobacillus ruminis*, *Parabacteroides merdae*, and *Eggerthella lenta*), including lactate-producing ones, were more abundant in females (Fig. 2). In the AD group, seven species exhibited sex-based differences, with predominance of commensals in males (*Blautia wexlerae* and *Blautia hansenii*) (Fig. 2). However, no sex differences were observed in the abundance of lactate-producing bacteria and butyrate-producing bacteria in the AD group. For CRC patients, 18 species showed sex-related differences, primarily in opportunistic pathogens. In the CRC group, lactate-producing bacteria and butyrate-producing bacteria were more abundant in females than in males, along with certain commensal bacteria (*P. merdae* and *Bacteroides vulgatus*). Collectively, sex-related differences in the gut microbiome were evident in HCs but diminished in patients with AD and CRC.

2. Effect of age on the gut microbiome composition

In the HC group, alpha diversity indices tended to decrease after the age of 55 years, with the Jackknife index showing a significant decrease (Supplementary Table 1, Supplementary Fig. 2). However, this age-dependent difference in alpha diversity vanished in both the AD and CRC groups, where the alpha diversity trend reversed in individuals under 55 years of age. Beta diversity analysis revealed significant clustering and separation by age only within the HC group (p=0.046), while the distribution was more scattered in the AD and CRC groups, indicating no age-dependent differences (Supplementary Fig. 2). Subsequently, age-dependent differences in taxonomic abundance at the phylum level were assessed. In the HC group, Actinobacteria and Firmicutes were less abundant in older individuals compared to those under 55 years of age, while Bacteroidetes were more abundant in older individuals. Only the difference in Actinobacteria abundance was statistically significant (p=0.023). However, these age-

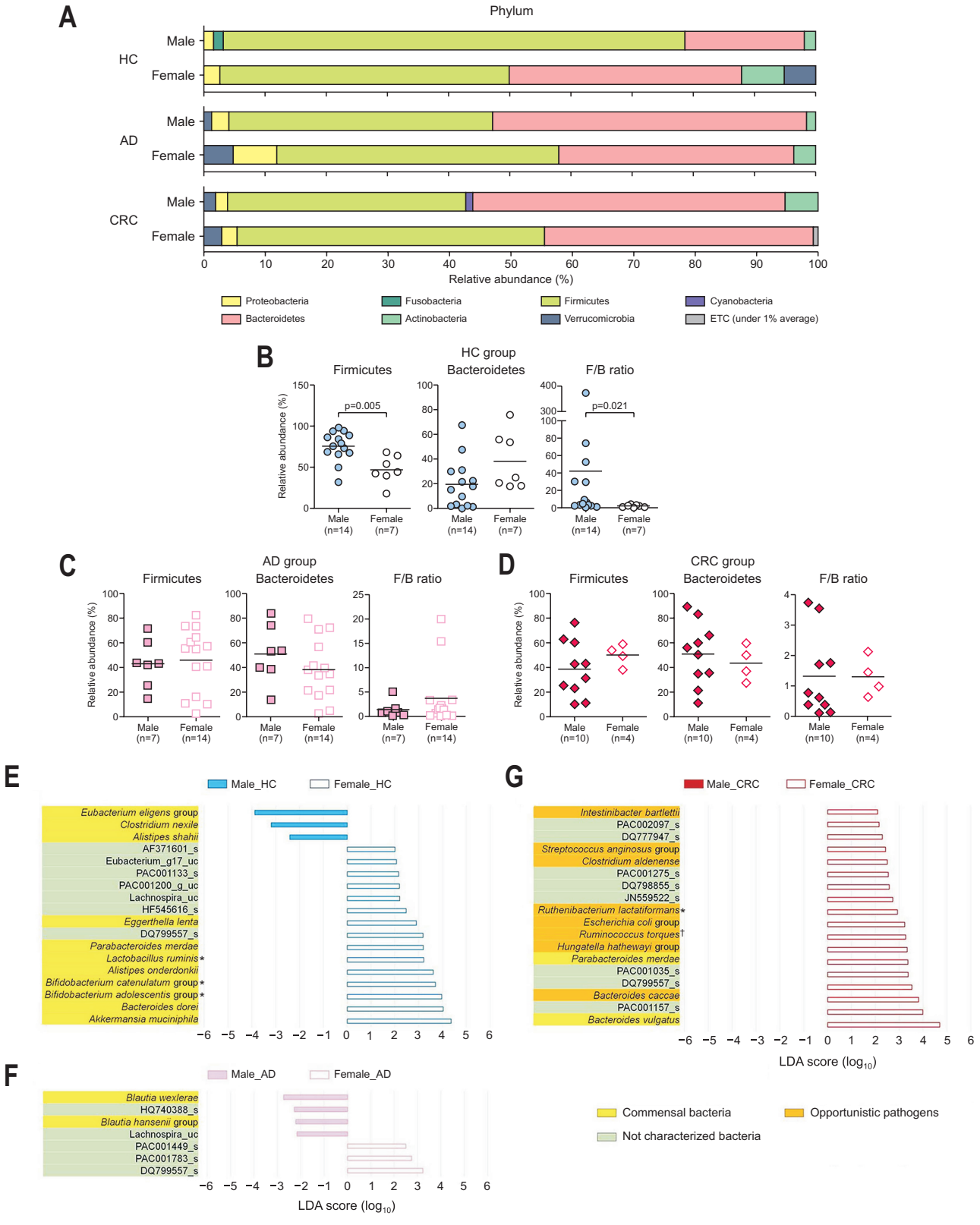


Fig. 2. Distribution of gut microbiome features according to sex in the HC, AD, and CRC subjects. (A-D) Gut microbiota compositions at the phylum level. (A) Bar graph for taxonomic composition. (B-D) Scatter plots for relative taxonomic abundance in HC (B), AD (C), and CRC (D) groups. Data are expressed as the mean±SEM. (E-G) Linear discriminant analysis (LDA) effect size (LEfSe) analysis according to sex in the HC (E), AD (F), and CRC (G) groups. HC, healthy control; AD, colorectal adenoma; CRC, colorectal adenocarcinoma; SEM, standard error of the mean; F/B, Firmicutes to Bacteroidetes. *Lactate-producing bacteria; †Butyrate-producing bacteria.

related taxonomic abundance differences observed in the HC group were not present in the AD and CRC groups (Supplementary Fig. 2).

Age-based differences in gut microbiome composition were assessed using the LEfSe method. In HCs, 13 species exhibited age-dependent differences, including eight commensal and five uncharacterized species. Among these, eight commensal bacteria were more abundant in individuals aged ≤55 years, while uncharacterized bacteria were predominant in those >55 years. Notably, butyrate-producing bacteria (*Agathobaculum butyriciproducens* and *Blautia faecis*) were three times more abundant in those <55 years (Fig. 3). In the AD group, 18 species displayed age-related differences, with one commensal, three opportunistic, and 14 uncharacterized species. Two opportunistic pathogens increased in abundance in individuals with AD aged <55 years, while commensal bacteria like *Bacteroides finegoldii* and the opportunistic pathogen *Phascolarctobacterium succinatutens* increased in those >55 years (Fig. 3). Among patients with CRC, nine species exhibited age-dependent differences, including three commensal, one opportunistic, and five uncharacterized species. Notably, one opportunistic pathogen (*Allisonella histaminiformans*) and two commensal bacteria (*Agathobacter rectalis* and *B. adolescentis* group) were more abundant in those <55 years compared to those ≥55 years (Fig. 3). Collectively, age-based differences in the gut microbiome composition observed in the HCs were not observed patients with AD and CRC.

3. Changes in the gut microbiome along the colorectal AD-CRC sequence

The gut microbiome diversity was further analyzed in relation to the HC-AD-CRC sequence. Beta diversity, which assesses clustering and similarity between samples, was examined using a PCoA plot at the species level. The results showed strong clustering and separation of the HC group from both the AD (p=0.002) and CRC (p=0.001) groups. However, the AD and CRC groups exhibited similar clustering, indicating no significant difference between them. Beta diversity analysis indicated significant differences among all comparison groups (p=0.001). Differences in taxonomic abundance at the phylum level were further analyzed using bar graphs and scatter plots. Firmicutes were significantly decreased in patients with AD (p=0.007) and CRC (p=0.003) compared to HCs. Conversely, Bacteroidetes showed a sequential increase in patients with AD (p=0.019) and CRC (p=0.005) compared to HCs (Fig. 4). The F/B ratio was significantly decreased in the AD (p=0.009) and CRC (p=0.003) groups compared to HCs. Fusobacteria significantly decreased in patients with AD (p=0.008) and exhibited a decreasing trend in patients with CRC compared to HCs. Actinobacteria significantly increased in patients with AD (p=0.024) and CRC (p=0.030) compared to HCs (Fig. 4).

Beta diversity analysis showed that the HC group was strongly clustered and separated from the AD (p=0.007) and CRC (p=0.001) groups in males. Furthermore, the AD and CRC groups were sporadically distributed; however,

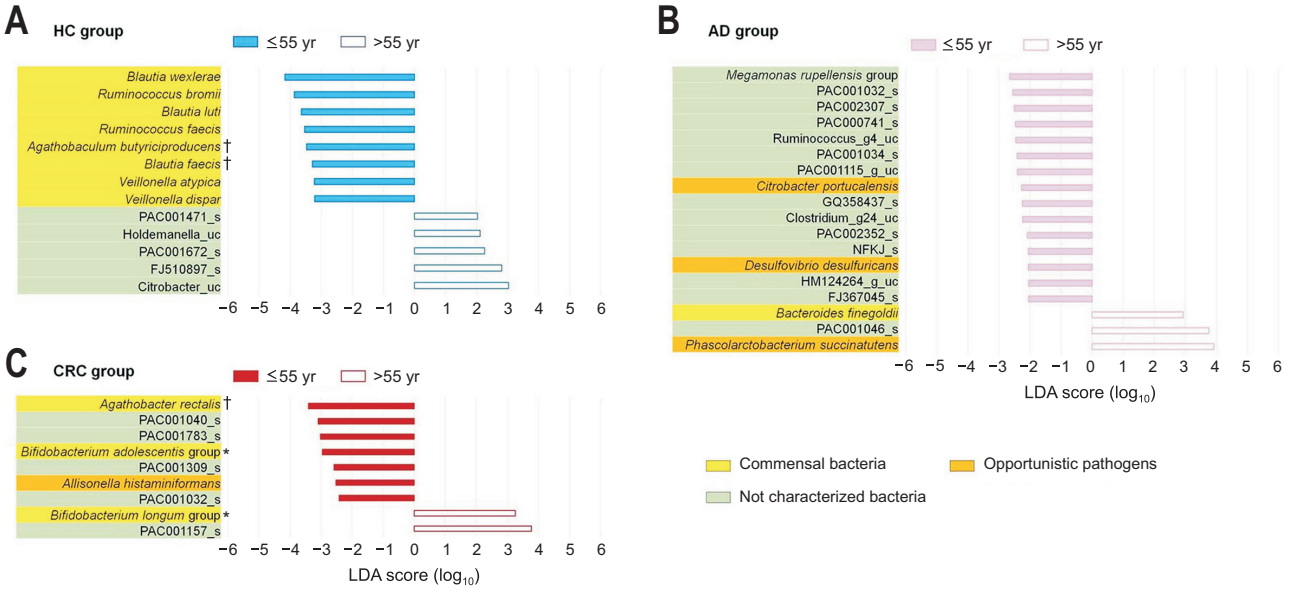
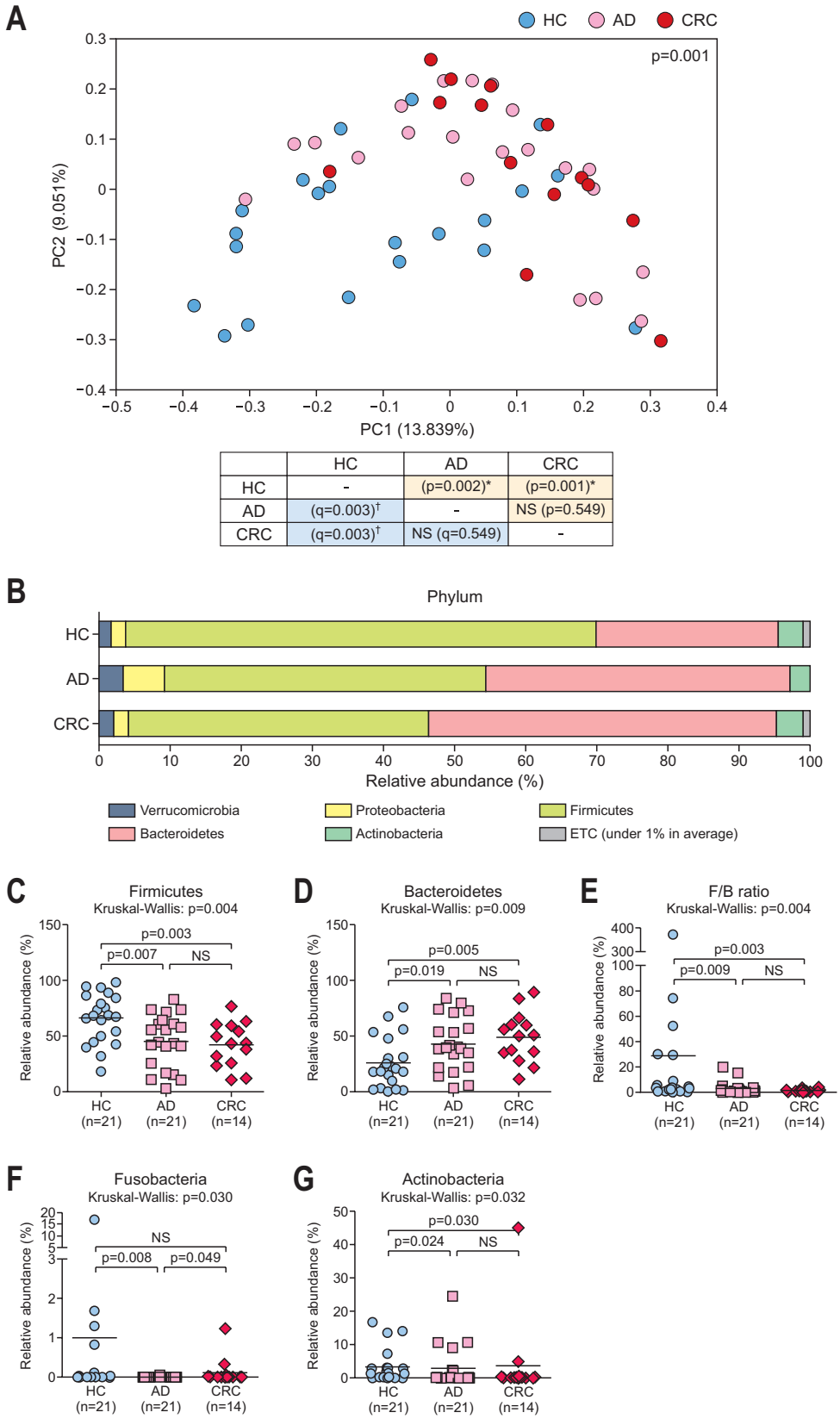


Fig. 3. Identification of taxonomic biomarkers. Linear discriminant analysis (LDA) effect size (LEfSe) analysis according to age in the HC (A), AD (B), and CRC (C) groups. HC, healthy control; AD, colorectal adenoma; CRC, colorectal adenocarcinoma. *Lactate-producing bacteria; †Butyrate-producing bacteria.



	HC	AD	CRC
HC	-	(p=0.002)*	(p=0.001)*
AD	(q=0.003)†	-	NS (p=0.549)
CRC	(q=0.003)†	NS (q=0.549)	-

Fig. 4. Distribution of gut microbiome features according to the HC-AD-CRC sequence in all participants. (A) Beta diversity of gut microbiota. (B-G) Gut microbiota compositions at the phylum level. Bar graph for taxonomic composition (B) and scatter plots for relative taxonomic abundance (C-G). Data are expressed as the mean±SEM. HC, healthy control; AD, colorectal adenoma; CRC, colorectal adenocarcinoma; SEM, standard error of the mean; NS, not significant. *p<0.01, †q<0.01.

no significant difference was observed among the males. Beta diversity analysis showed significant differences in all comparison groups in the males (p=0.002). Taxonomic bar graphs of the male patients with AD and CRC and HCs are shown in Supplementary Fig. 3. Firmicutes significantly decreased sequentially in patient with AD (p=0.004) and CRC (p=0.001) than in the HCs. Conversely, Bacteroidetes significantly increased in patients with AD (p=0.009) and CRC (p=0.005) than in the HCs. The F/B ratio was significantly lower in patients with AD (p=0.007) and CRC (p=0.002) than in the HCs. Fusobacteria significantly decreased in patients with AD (p=0.029) and showed a decreasing tendency in patients with CRC than in the HCs. Actinobacteria significantly increased in patients with AD (p=0.036) and showed an increasing tendency in patients with CRC than in the HCs (Supplementary Fig. 3). However, in female patients, there was no significant difference in beta diversity at the species level or taxonomic abundance at the phylum level (Supplementary Fig. 4).

4. Identification of specific gut microbiome according to CRC progression

LEfSe analysis revealed differences between HCs and patients with AD and CRC. In total, 57 species (15 commensal, 3 opportunistic, and 39 uncharacterized bacteria) were altered in the AD group, and 74 species (14 commensal, 5 opportunistic, and 55 uncharacterized) were altered in the CRC group (Supplementary Fig. 5). Among these, 22 species (10 commensal, 1 opportunistic, and 17 uncharacterized) were commonly altered in both disease groups.

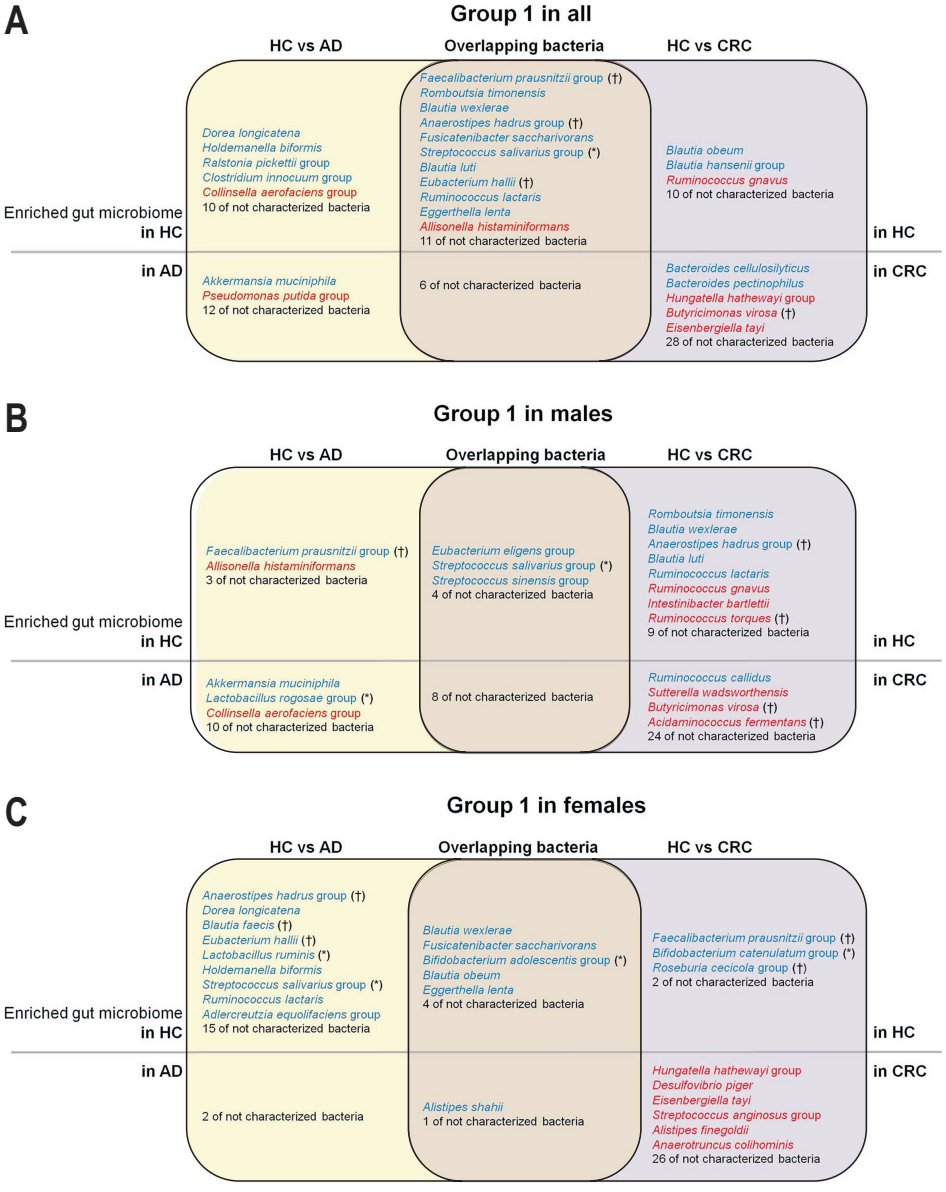


Fig. 5. Venn diagrams based on linear discriminant analysis (LDA) effect size (LEfSe) data (Supplementary Figs. 5-7). (A) Identification of bacteria exhibiting changes after colorectal disease progression compared to HC control in all participants. (B) Identification of bacteria exhibiting changes between male subjects with colorectal disease progression and male HCs. (C) Identification of bacteria exhibiting changes between female subjects with colorectal disease progression and female HCs. HC, healthy control; AD, colorectal adenoma; CRC, colorectal adenocarcinoma. *Lactate-producing bacteria; †Butyrate-producing bacteria.

Notably, 10 commensal bacteria, including lactate-producing *Streptococcus salivarius* and butyrate-producing *Faecalibacterium prausnitzii*, *Anaerostipes hadrus*, and *Eubacterium hallii*, which were abundant in HCs, decreased in both AD and CRC groups. Additionally, 29 species were specific to AD, and 46 species were specific to CRC (Fig. 5 and Supplementary Fig. 5).

When considering sex differences, in male patients, 33 species (6 commensal, 2 opportunistic, and 25 uncharacterized) were altered in the AD group, and 60 species (9 commensal, 6 opportunistic, and 45 uncharacterized) were altered in the CRC group (Supplementary Fig. 6). Among these, 15 species (3 commensal and 12 uncharacterized) were commonly altered in both diseases. Notably, the commensal bacteria *S. salivarius*, which produces lactate and was abundant in male HCs, decreased in male patients with AD and CRC. Furthermore, 18 species were specific to AD, and 45 species were specific to CRC in males (Fig. 5 and Supplementary Fig. 6).

In female patients, 37 species (15 commensal and 22 uncharacterized) were altered in the AD group, and 48 species (9 commensal, 6 opportunistic, and 33 uncharacterized) were altered in the CRC group (Supplementary Fig. 7). Among these, 11 species (6 commensal and 5 uncharacterized) were commonly altered in both diseases. Notably, the commensal bacteria *B. adolescentis*, which produces lactate and was abundant in female HCs, decreased in females with AD and CRC. Furthermore, 28 species were specific to AD, and 37 species were specific to CRC in females (Fig. 5 and Supplementary Fig. 7).

5. Identification of sex-specific gut microbiome in younger HC group

We additionally performed LEfSe analysis according to sex in HC group below 55 years of age. Fourteen species (five commensal, one opportunistic, and eight uncharacterized) in younger HC group showed sex-based differences. Interestingly, the *B. catenulatum* group belonging to lactate-producing bacteria was highly abundant in females compared to males (Fig. 6).

DISCUSSION

Our study showed that females in the HC group had more lactate-producing bacteria (*B. adolescentis*, *B. catenulatum*, and *L. ruminis*) than the males in the HC group. Furthermore, younger subjects (≤55 years) had more butyrate-producing bacteria (*A. butyriciproducens* and *B. faecis*) than the older patients (>55 years) in the HC group. These sex- and age-dependent differences in HC disappeared in the AD and CRC groups, suggesting

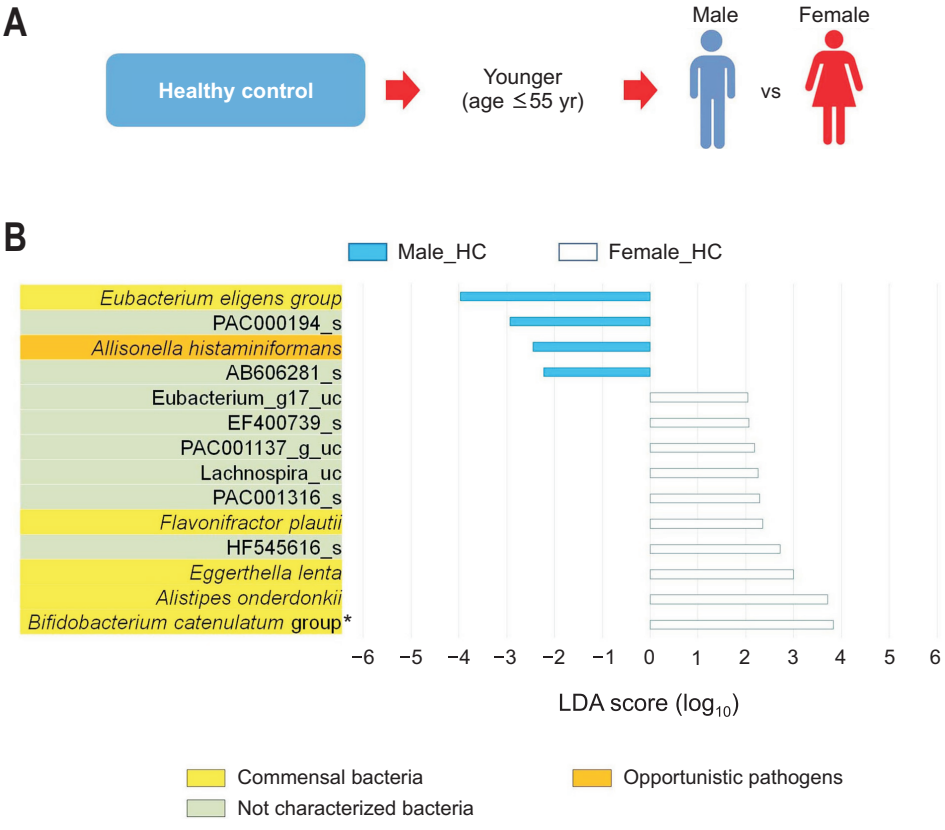


Fig. 6. Linear discriminant analysis (LDA) effect size (LEfSe) analysis according to sex in younger healthy control (HC) than 55 years of age. (A) Analysis scheme. (B) LEfSe analysis according to sex in younger HC subjects. *Lactate-producing bacteria.

that the gut microbiome diversity in patients with AD and CRC is strongly clustered in a different direction. In addition, commensal bacteria, including lactate-producing (*S. salivarius*) and butyrate-producing (*F. prausnitzii*, *A. hadrus*, and *E. hallii*) bacteria, were more abundant in the HC group than in the AD and CRC groups. Furthermore, lactate-producing bacteria (*B. catenulatum* group) were highly abundant in females compared to males in younger subjects of the HC group, indicating preventative role of lactate- and butyrate-producing bacteria in the colorectal carcinogenesis, especially in young age and females.

In our study, patients with AD and CRC exhibited lower Firmicutes levels and higher Bacteroidetes levels compared to the HCs. Moreover, Actinobacteria and Firmicutes levels were higher in younger patients than in older patients within the HC group. Actinobacteria, primarily represented by the genus *Bifidobacterium*, is a commensal probiotic in the human intestine associated with human health.[27] Firmicutes and Bacteroidetes, representative bacterial phyla that exhibit differences in relation to obesity, displayed sex-based differences in the HC group. However, following body mass index analysis, no significant sex-based differences were observed within the group (data not shown). The F/B ratio, which involves the dominant phyla Firmicutes and Bacteroidetes, has been linked to various pathological conditions, including CRC.[28] Obese individuals and animals tend to have higher F/B ratios compared to normal-weight individuals.[29] It is worth noting that the F/B ratio has been associated with an increased risk of CRC in a significant portion of patients.[10,30] Unlike obesity, inflammatory bowel disease is reported to have a low F/B ratio.[31] Interestingly, in this study, a decrease in F/B ratio in the AD/CRC group compared to the HC group was observed in males, but no such change was observed in females. It is believed that it may be difficult to accurately represent the physiology of the biological situation observed in males due to the presence of more complex factors, including hormones, and differences in immune responses in females compared to males.[32,33] However, the gut microbiome distribution can vary widely among different studies, leading to contradictory findings. For instance, some studies have reported lower Firmicutes abundance in patients with inflammatory bowel disease like Crohn's disease and ulcerative colitis compared to HCs.[34] Firmicutes were also found to be dramatically decreased in older Chinese patients (>50 years) compared to younger patients.[12,13] Additionally, the relative abundances of Bacteroidetes, Proteobacteria, and Verrucomicrobia were reported to be higher in older individuals.[13] Overall, these results suggest that the distribution of specific beneficial bacteria at the species level might hold more significance than the F/B ratio alone.

While Fusobacteria are commonly found in the human oral and gastrointestinal tracts, some species of Fusobacteria can act as opportunistic pathogens. In the present study, the abundance of Fusobacteria was lower in patients with AD and CRC compared to HC, and higher in patients with CRC compared to those with AD. As a result of each case analysis, several species belonging to the phylum Fusobacteria were identified, including *Cetobacterium somerae*, an uncharacterized *Cetobacterium* species, and several species from the *Fusobacterium necrogenes*, *Fusobacterium nucleatum*, and *Fusobacterium varium* groups. Strangely, the abundance of *F. nucleatum*, previously associated with CRC,[35] was found to be low (less than 0.2%) and present only in three cases out of 14 cases in the CRC group (Supplementary Dataset). Additionally, other species of bacteria were found to be mixed in the HC, AD, and CRC groups. However, similar study showed that *F. necrogenes*, *Fusobacterium mortiferum*, *F. varium*, and *Fusobacterium ulcerans* were not associated with CRC.[36] Thus further studies are needed in the large cohort.

Butyrate, a major SCFA, plays a vital role in digestive health and the prevention of diseases such as CRC, inflammatory bowel disease, and obesity.[37] In our study, we observed differences in the gut microbiome that are relevant to these diseases. Specifically, we found that phylotypes closely related to Bacteroides were more prevalent in CRC patients, while butyrate-producing bacteria like *Faecalibacterium* and *Roseburia* were less abundant in CRC patients compared to HCs.[38] Additionally, there are sex-specific differences in the butyrate-producing gut microbiome, often referred to as "microgenderomes."[39] Lactic acid is typically considered an intermediate substance produced by gut microorganisms and serves as a source of SCFAs, particularly butyrate.[40] Although lactic acid-producing bacteria are commonly associated with Lactobacillales, certain bacteria from the genus *Bifidobacterium* also produce lactic acid as a major product of carbohydrate metabolism.[41] Lactic acid-producing bacteria, the primary probiotics in the intestine, predominantly colonize from the duodenum to the end of the ileum.[42] They could boost immunity, improve gastrointestinal function, enhance resistance to obesity, increase antioxidant abilities, and reduce blood glucose and cholesterol levels.[42] Additionally, numerous studies have suggested their potential anti-cancer effects.[42] In a previous study, a higher abundance of the lactic acid-producing bacteria, especially *Lactobacillus murinus* species was observed in male mice treated with azoxymethane and dextran sodium sulfate and supplemented with E2 compared to male mice treated with azoxymethane and dextran sodium sulfate alone.[8] In our study, we observed clear differences in the bacterial distribution of the gut microbiome in females,

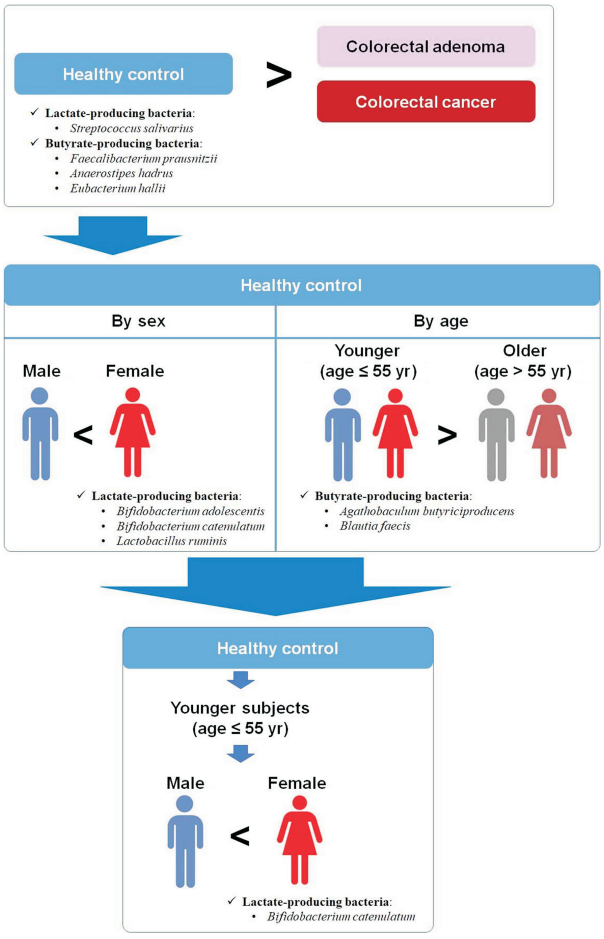


Fig. 7. Summary of the gut microbiome features depending on sex, age, and colorectal carcinogenesis progression based on human fecal sample analysis.

with commensal bacteria being predominant in the HC group and opportunistic pathogens more prevalent in the disease group, especially in CRC (Fig. 5). Various lactic acid-producing bacteria (such as *L. ruminis*, *S. salivarius*, *B. adolescentis*, and *B. catenulatum*) and butyrate-producing bacteria (such as *A. hadrus*, *B. faecis*, *E. hallii*, *F. prausnitzii*, and *Roseburia cecicola*) were more abundant in the HC group compared to the AD and CRC groups in females but not in males. Furthermore, lactate-producing bacteria (*L. ruminis*, *B. adolescentis*, and *B. catenulatum*) were more prevalent in females than in males. Overall, our findings suggest that various gut bacteria undergo changes in the HC-AD-CRC sequence, with sex and age playing significant roles. These differences in the gut microbiome related to sex may contribute to the earlier onset and higher incidence of CRC in males compared to females. Since the results of this study alone cannot determine a causal relationship, further research is needed to determine the causal relationship between disease progression depending on sex and the commensal bacteria, including butyrate-producing and lactate-producing bacteria discovered in this study.

This study has several limitations. First, this study was conducted with a small sample size due to the challenges in enrolling patients, considering various factors such as sex, age, diet, and environmental conditions. Moreover, healthy subjects, especially older individuals, who visited the hospital solely for colonoscopy were more likely to refuse enrollment in stool studies. Thus to increase the enroll number was very difficult. For this reason, there was a difference in the average age of the HC group and the AD/CRC group. We have a plan of further enrollment in each group to compare the distribution of gut microorganisms within similar age groups. Second, because groups were compared at the endpoint, it was not possible to confirm changes in the gut microbiota according to the disease progression. Despite these limitations, we comprehensively researched gut microbiome in terms of CRC carcinogenesis depending on age and sex (Fig. 7), and suggested preventative role of lactate- and butyrate-producing bacteria in colorectal carcinogenesis, especially in young age and females.

CORRECTION

This article was corrected on February 26, 2024, for an author list.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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AUTHOR CONTRIBUTIONS

Study concept and design: N.K., Y.J.S. Data acquisition: N.K., H.Y. Data analysis and interpretation: C.S. Drafting of the manuscript: C.S. Critical revision of the manuscript

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SUPPLEMENTARY MATERIALS

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