The gut microbiome alteration in colorectal cancer, compared by pre and postoperative change

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The gut microbiome alteration in colorectal cancer, compared by pre and postoperative change

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ABSTRACT

The gut microbiome alteration in colorectal cancer, compared by pre and postoperative change

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Colorectal cancer (CRC) is one of the leading causes of cancer-associated mortality worldwidely. Emerging evidence has shown that intestinal dysbiosis is closely associated with CRC incidence rates. Certain colon microbes in the typical colon flora may influence the microenvironment, creating a favorable environment for cancer development. However, gut microbiota dysbiosis remains poorly understood.

This study aimed to clarify how gut microbiota changes affect the development and progression of CRC. The differences in colon microbiome composition was compared between serial changes in each CRC patient by analyzing preoperative and postoperative stool.

Human fecal samples were collected preoperatively and 3–6 months postoperatively from 40 CRC patients who underwent curative surgery at Severance Hospital. Whole-genome shotgun sequencing (WGS) was performed on microbial genomic DNA extracted from fecal samples. Operational taxonomic units (OTUs), alpha diversity, beta diversity, and bacterial communities were evaluated at genus and species levels in human fecal samples before and after surgery. Principal coordinate analysis (PCoA) and Differential abundance analysis (DA) were also performed. Furthemore, KEGG enrichment analysis of differentially expressed genes (DEGs) was performed to evaluate immunoglobulin A (IgA) protease status from RNA sequencing data.

This study shows microbiome differences between pre- and post-operative status in human fecal samples. Alpha diversity and OTUS were significantly decreased in post-operative samples compared with the levels in pre-operative samples (p<0.05). Between pre-operative and post-operative samples, there was a significant difference in terms of β-diversity (p =0.006) in the genus level. In prticular, greater changes in alpha diversity were observed in strains with fewer antibiotic resistance gene (ARG) than in strains with many ARG. PCoA and DA also showed pre-operative and post-operative microbiome componenet differences. *Fusobacterium*, *Prevotella,* and *Peptostreptococcus* in the genus level were abundant before surgery, whereas *Sellimonas intestinalis* was highly observed after surgery. Most of the strains with high copy numbers of the IgA protease gene were known pathogen strians such as *Escheria, Rothia, Salmonella, Haemophius, and Helicobacter.*

This study draws an initial point that gut microbiota imbalance is a risk factor of CRC. *Fusobacterium*, *Prevotella* seem to be related to CRC, and the degree of inclusion of ARG or IgA protease also appears to affect changes in microbiome composition. Gut microbiota change may provide a new therapeutic avenue for CRC patients.

Keywords: Microbiota, Gut microbiome, Colorectal cancer

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I. INTRODUCTION

Colorectal cancer (CRC) is still one of the leading causes of cancer-associated mortality [1]. To prevent CRC, many studies are being held, however, as genetic syndromes account for a minority of cases of CRC, controlling environmental or lifestyle risk factors such as obesity and diet modification are focused on nowadays [2,3]. The colon is the most heavily colonized section of the digestive tract, and it has been estimated that this organ contains approximately 70% of the estimated human microbiome. Dietary habits and lifestyle are known risk factors in CRC, and they may also modulate gut microbiota. Thus, a possible hypothesis is that certain colonic microbes or alteration of the typical resident colonic flora may influence to microenvironment that is favorable to cancer development [4]. Recently, a growing number of studies reported specific alterations in the gut microbiome associated with CRC and explored its value for CRC screening. For details, *F. nuleatum* and *B.fragilis* are the most representative microbiome related to a negative impact on survival outcomes [5]. In the long run, a better knowledge of the relationships between the microbiota and the origin and progression of CRC may open novel opportunities for the development of therapies targeting the microbiome. In this regard, the development and

use of prebiotics, probiotics, specific antibiotics, phage therapies, or the transplantation of whole microbiomes may bring new tools for the prevention and treatment of CRC [6].

Thus, by detecting certain specific microbiomes, screening CRC may be easier, and if CRC is detected in an earlier stage, the treatment will have a higher success rate. The current study will analyze serial colonic microbiome composition change in each CRC patient by analyzing pre- and post-operative stool. These changes will be compared with clinicopathological findings to figure out how they affect CRC development and its progression.

II. MATERIALS AND METHODS

1. Sample collection

Human fecal samples were collected from 40 colorectal cancer patients at baseline (before the surgery within 1 week) and 3 or 6 months after surgery. All stool samples were collected more than 35 g were placed in cryotubes and stored at −80 °C.

Formalin-fixed paraffin embedded (FFPE) tissues were obtained from from the Severance Tissue Bank, in the form of 4 μm thick sections on slides. Total RNA from FFPE tisseus was used for RNA sequencing.

This study was approved by Yonsei University Health System (IRB: 4-2019-0676).

2. Microbial DNA extraction and whole genome shotgun sequencing (WGS)

Microbial DNA was extracted using the PowerSoil DNA Isolation Kit PowerSoil DNA Isolation Kit (Qiagen, Valencia, CA, USA, Cat no. 12888-100) following the manufacturer's instructions. DNA samples were quantified using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA samples were stored at −80 °C until further processing.

Shotgun metagenomic paired-end libraries was constructed from 50 ng of pure DNA. The indexed libraries were sequenced using 2 × 150 bp paired-end kit on the Illumina Novaseq platform (Illumina, San Diego, CA, USA). The amount of raw sequencing data was 6 Gb for pre- and post-surgical stool samples of CRC patients. Microbial population analysis workflow is illustrated in **Figure 1**.

3. RNA sequencing

RNA was extracted from FFPE unstained slides using SureSelectXT RNA Direct Library Preparation kit (Agilent Technologies, Inc., Santa Clara, CA, USA). following manufacturer's protocols. Libeary quality was confirmed using an Agilent 2200 Tapestation system with the High Sensitivity D1000 screen tapes (Agilent Technologies, Inc., Santa Clara, CA, USA). The indexed libraries were then sequenced using Illumina NovaSeq (Illumina, Inc., San Diego, CA, USA)

4. Bioinformatics analysis

All RNA sequencing datasets was aligned to the human genome reference (GRCh38). Differentially Expressed Genes (DEG) and the Gene Ontology (GO) was identified from RNA sequencing data. For quality control of data, Knead Data software was used on the Fastq raw data based on Trimmomatic and Bowtie2 de-hosting [7]. Taxonomic profiling of the sequenced samples was analyzed using MetaPhlAn2 (version 2.6.0) [8]. Each sample will be run through the metaphlan.py script to generate the kingdom-specific taxonomic profile per sample, using the flag to generate relative abundances and estimated read counts. Functional profiling of the microbial community was evaluated using HUMAnN2 (version 0.11.1) [9]. Outputs was normalized to relative abundances and finally, merged into individual tables for all samples. All data was visualized using both Graphpad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA) and Rstudio software version 2023.09.1+494.pro2 (RStudio, Boston, MA, USA).

5. Statistical analysis

For the statistical analysis of the bacterial abundance data, compositional data analysis methods will be used. Features with a false discovery rate (FDR) of less than 10% will be considered significant. Statistical analysis was carried out by using Graphpad Prism 8 software (GraphPad Software Inc., San Diego, CA, USA). All data are presented as the means ± standard deviation (SD). A P-value < 0.05 is considered significant.

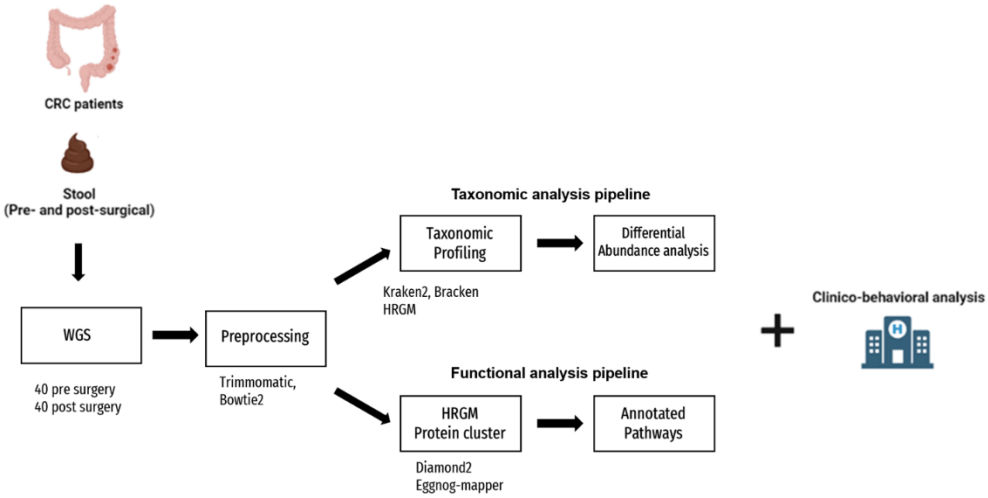


Figure 1 Study design and experimental protocol

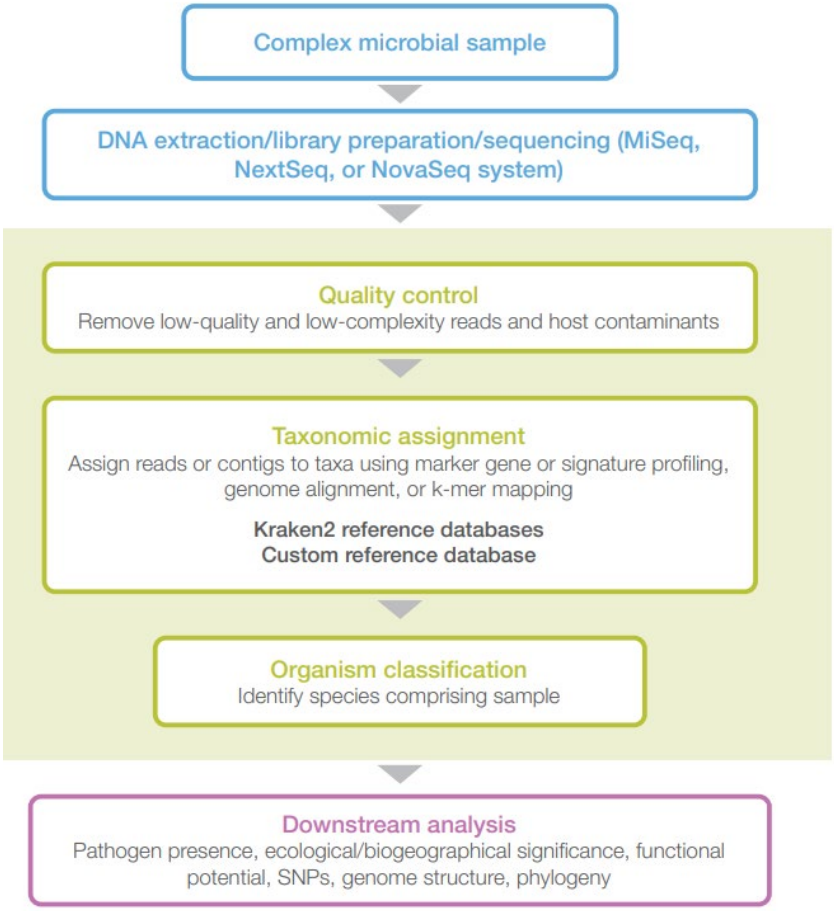


Figure 2 Microbial population analysis workflow

III. RESULTS

1. Patient characteristics

Patient characteristics are summarized in **Table 1**. A total of 40 patients were included, and their fecal samples were collected before and after surgery. Their median age was 60 years. As for staging, 17.5% (*n* = 7) of patients were classified as stage I, 42.5% (*n* = 17) as stage II, 37.5% (*n* = 15) as stage III, and 2.5% (*n* = 1) as stage IV. Cohort was comprised of left-sided colon cancers (72.5%; *n*= 29) and microsatellite stable cancers (100%). Of the 40 patients with CRC, mutations in KRAS were found in 35% (*n* = 14), in NRAS in 2.5% (*n* = 1), and in BRAF in 2.5% (*n* = 1). The carcinoembryonic antigen (CEA) level decreased from 4.52 ng/mL to 1.83 ng/mL after surgery.

Table 1. Patient characteristics

Characteristics	***n* = 40**	**(%)**
Sex		
Male	23	(57.5)
Female	17	(42.5)
Median age (year)	60	
Location of tumor		
Right side	11	(27.5)
Left side	29	(72.5)
Differentiation		
Well differentiated	2	(5.0)
Moderately differentiated	38	(95.0)
Stage		
I	7	(17.5)
II	17	(42.5)
III	15	(37.5)
IV	1	(2.5)
Tumor size (cm)	3.5 ± 1.87	
MSI		
MSS	40	(100)
***KRAS* mutation**		
Wild-type	26	(65.0)
Mutation	14	(35.0)
***NRAS* mutation**		
Wild-type	39	(97.5)
Mutation	1	(2.5)
***BRAF* mutation**		
Wild-type	39	(97.5)
Mutation	1	(2.5)
BMI (kg/㎡)	24.75 ± 3.45	
CEA (ng/mL)		
Pre-operative	4.52 ± 2.61	
Post-operative	1.83 ± 2.49	

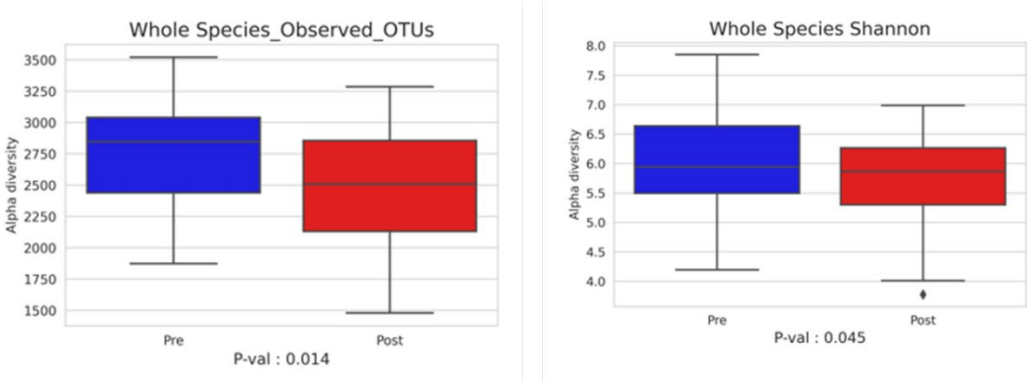
Abbreviation: MSI; Microsatellite instability, MSS; Microsatellite stability, BMI; Body mass index, CEA; Carcinoembryonic antigen,

2. Diversity analysis

In order to identify potential differences within-sample diversity, known as alpha-diversity was calculated. Alpha diversity, which characterizes diversity at a local scale, delineates the species richness within a functional community. In the species levels, measures of richness and Shannon alpha diversity exhibited significant decreases in post-operative samples (**Figure 3A**). In the richness of the genus level, there was a significant decrease in post-operative samples compared to the pre-operative samples (**Figure 3B**).

The beta diversity which is known as between-sample diversity. To evaluate microbiota changes in pre- and post-operative samples, beta diversity was analyzed by conducting principal coordinate analysis (PCoA). When using Bray-Curtis dissimilarity and Weighted unifrac for group comparison (**Figure 3C**), no differences of bacterial communities were observed between pre- and post-operative samples in the species level. Aitchison dissimilarity matrix was higher in post-operative samples than pre-operative sample (**Figure 3D**, $p = 0.002$). On the contrary, the Bray-Curtis dissimilarity in the genus level was lower than in post-operative samples than pre-operative sample, as shown **Figure 3E**.

(A)



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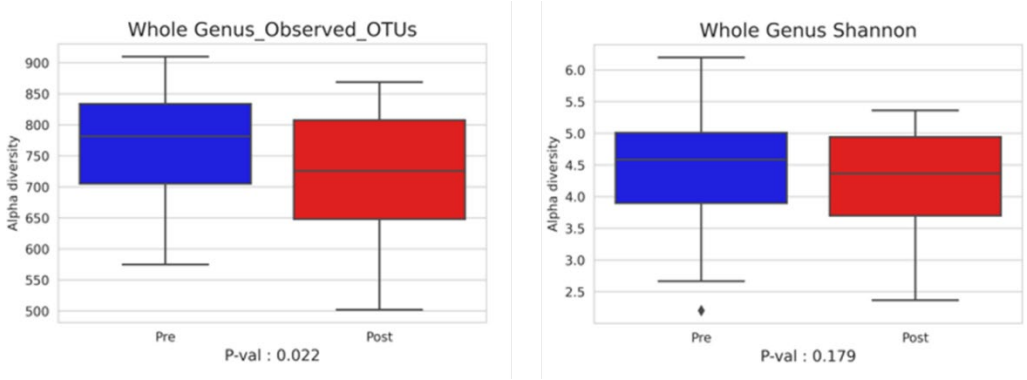
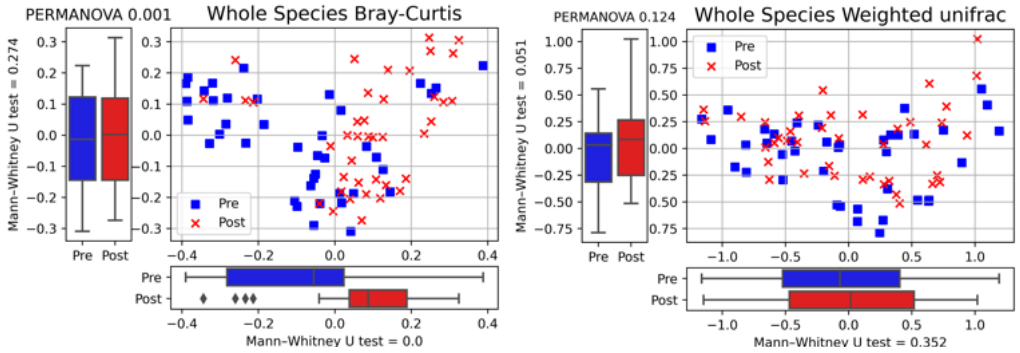


(C)



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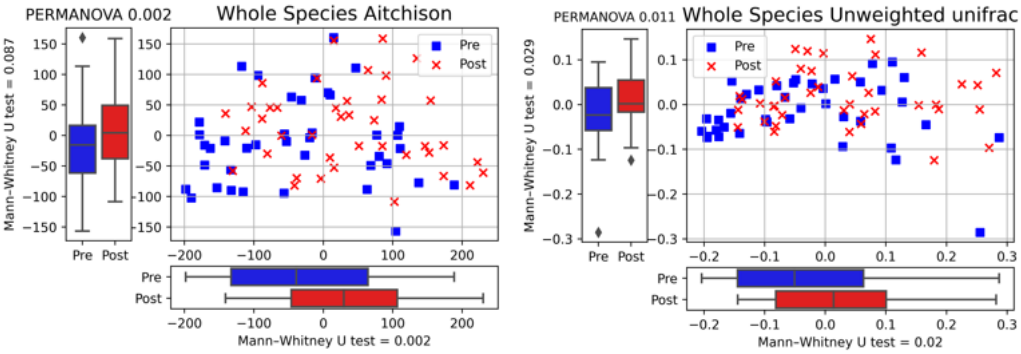

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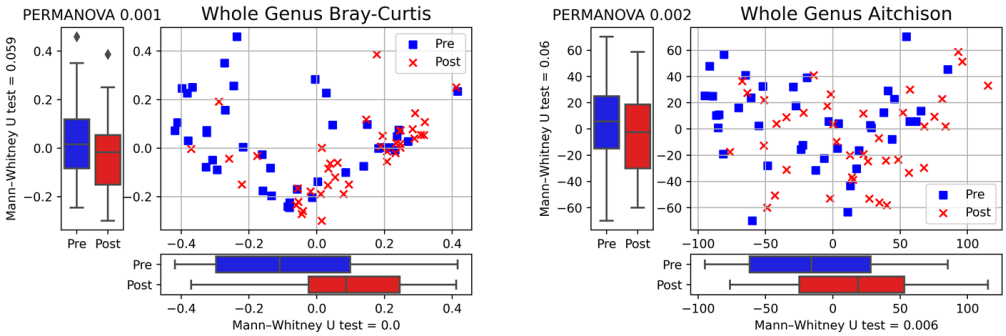


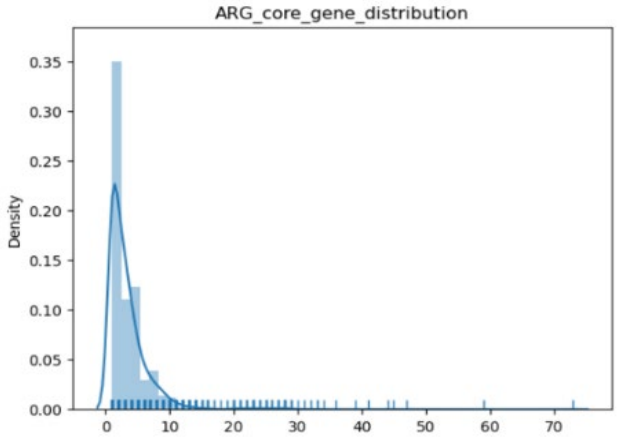
Figure 3 Diversity analysis in genus and species level before and after surgery

(A) Alpha diversity in terms of OTUs in the genus and species levels, (B) Alpha diversity in terms of Shannon in the genus and species levels. Group differences in β-diversity at the genus and species levels; (C) Bray-Curtis and unifrac in the species level, (D) Aitchison and unifrac in the species level, and (E) Bray-Curtis and unifrac in the genus level

3. Antibiotic resistance gene (ARG) analysis

Alpha diversity at the species level was re-classified by Antibiotic Resistance Gene (ARG) status. Distribution of ARG is shown in **Figure 4A**. Species that have more than 4 ARG were categorized as “High ARG species” while those with less than 4 ARG were considered as “Low ARG species”. Results shows that high variations in OUTs among "Low ARG species," while demonstrating comparatively lower alpha diversity differences in "High ARG species" (**Figure 4B**). When examining Shannon in the species level, a significant difference in low ARG abundance between post-operative samples compared to pre-operative samples were also found. ($p = 0.016$).

(A)



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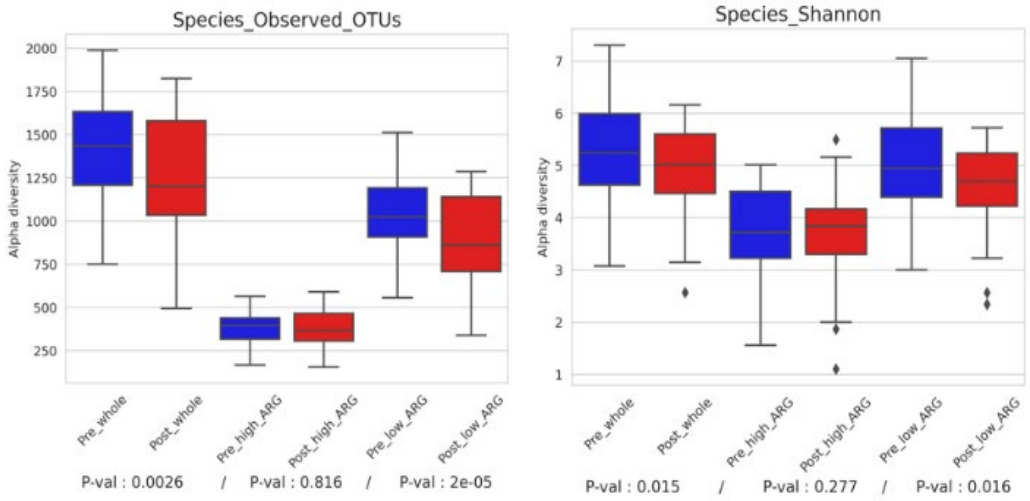


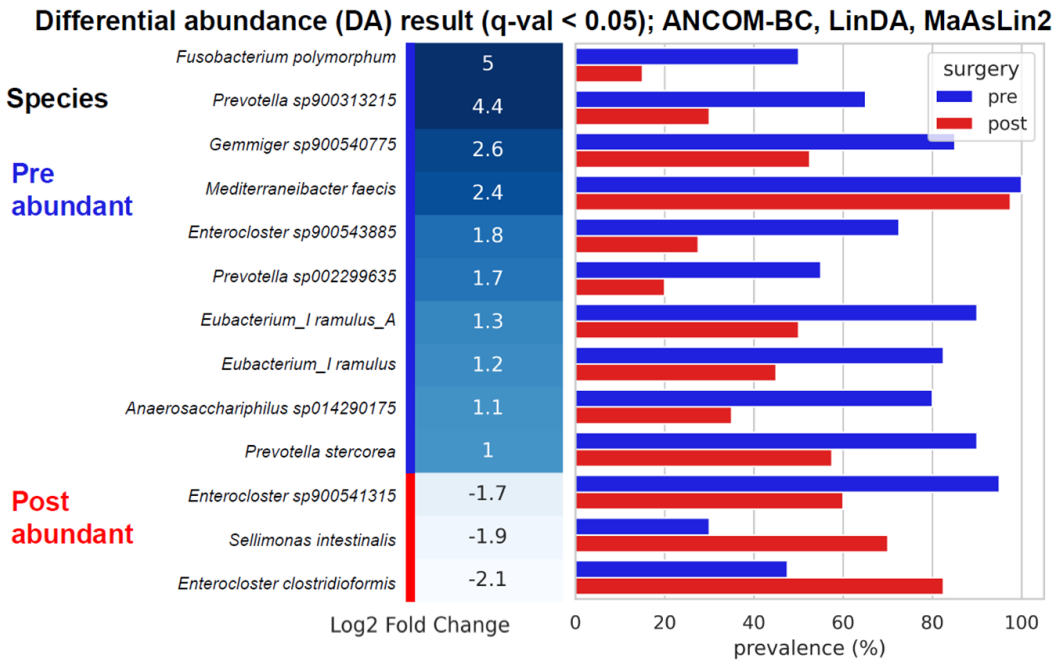
Figure 4 Antibiotic resistance gene (ARG) analysis

(A) Distribution of ARG core genes, (B) Alpha diversity in terms of OTUs and Shannon in the species levels based on ARG status.

4. Differential abundance analysis

Differential abundance analysis (DA) aims to find the differences in the abundance of each taxon between two classes of subjects, assigning a significance value to each comparison. Various perspectives were explored using Analysis and Comparison of Maps (ANCOM-BC), MaAsLin2, and LinDa tools. As shown in **Figure 5A**, species differed significantly in abundance on a log2 fold change scale between pre- and post-operative samples were analyzed. In intra-cross-validation using species-level taxonomic relative abundances, area under the curve (AUC) score was 0.858 (**Figure 5B**). Next, the metagenomic classification was conducted by SIAMCAT (Statistical Inference of Associations between Microbial Communities And host phenoTypes). Of these, metabolic features that were significantly different between pre- and post-operative samples are represented in **Figure 5C**. *Prevotella, Porphyromonas,* and *Fusobacterium* were the most abundant species present in pre-operative samples compared to post-operative samples. *Lactiplantibacillus, Entreocloster, Enterobacter,* and *Lawsonibacter* were more common in post-operative samples (**Figure 5D**). Through this analysis, the majority of taxa that were abundant before surgery were verified as being associated with CRC.

(A)



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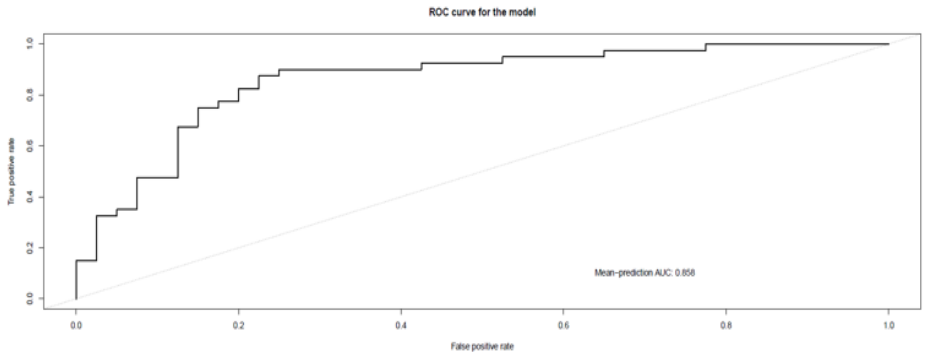
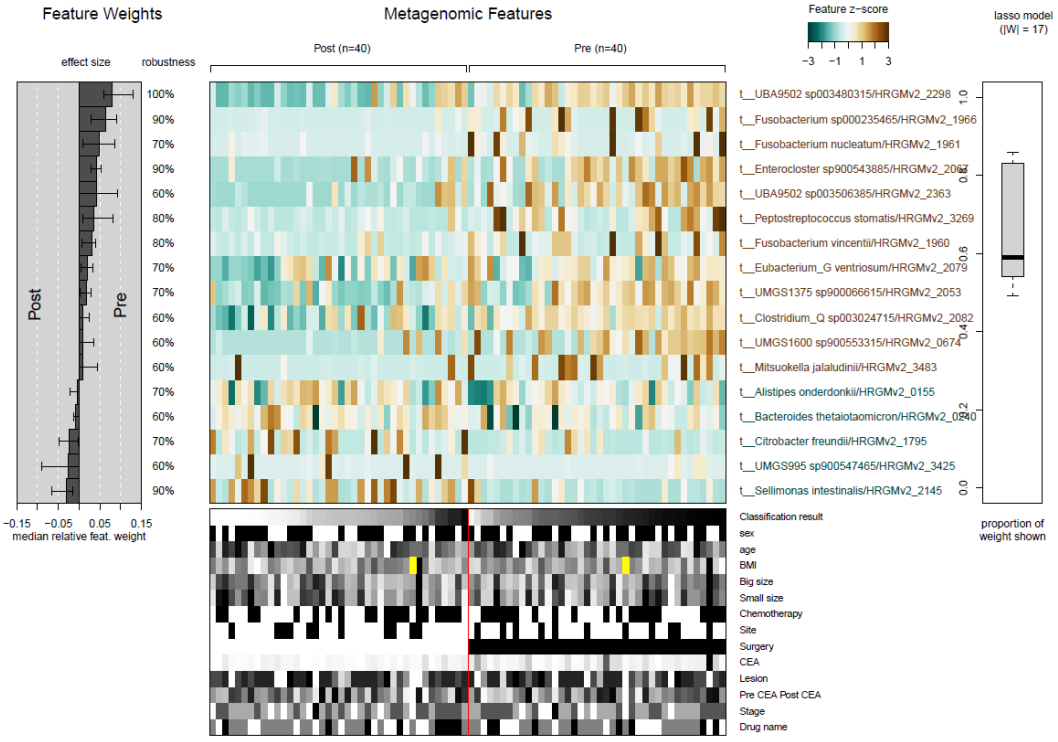


Species, Mean AUC : 0.858

(C)



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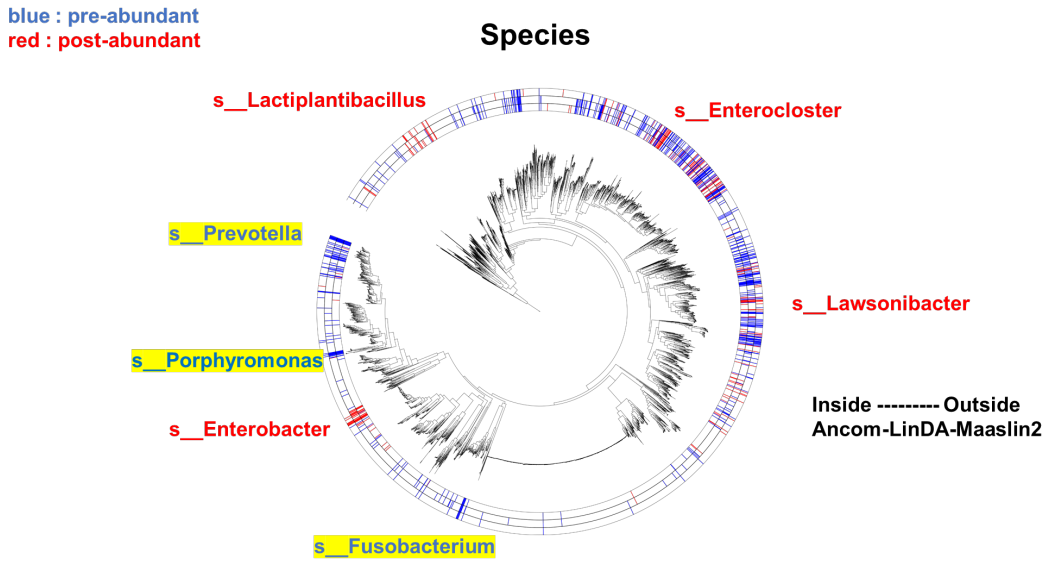


Figure 5 Differential microbiota and metabolic features between pre- with CR and post-operative samples

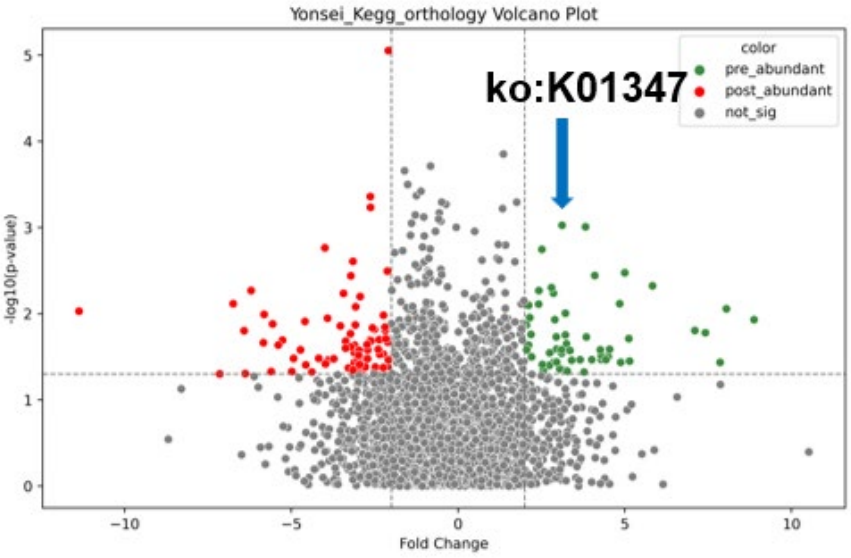
(A) Differential abundance of microbial composition (log2 fold change), (B) random forest machine learning classification model of pre-operative vs post-operative using all microbiome species, (C) heatmap of the number of taxonomic biomarkers identified from species profiles, (D) cladogram of the hierarchy among discriminative taxa in the species.

5. Functional profiling

Functional profiling is also used to predict and interpretate microbiome. After preprocessing WGS read data, alignment is performed to the gene sequence or protein sequence. Then, functional analysis was conducted through the previous known database of each protein or genes. Genes matched with KEGG genes and KEGG orthology (KO) were calculated. The volcano plot showed that there was significant change the function of microbial communities between pre-operative and post-operative samples (**Figure 6A).** Result shows immunoglobulin A **(**IgA)-specific serine endopeptidase (ko:K01347) is abundant in pre-operative samples (**Figure 6B**). Yonsei IgA difference was calculated by subtracting the post-IgA count from the pre-IgA counts. Most of samples showed a lot of IgA protease in pre-surgery rather than post-surgery **(Figure 6C)**. IgA is an antibody that plays a role in immune function of mucus membranes and it is reported that patients with IgA deficiency can have an increased risk of cancer. [10-12] IgA protease has a function of IgA-specific serine endopeptidase, which is to degrade IgA antibodies, and it plays a crucial part of the immune system's defense against pathogens.[13] **Figure 6D** shows that genus each species is located in. It shows the average copy number of the IgA protease gene of the species belonging to the genus in descending order. There were many well-known pathogens such as *Rothia, Salmonella, Haemaphilus, Helicobacter* and *Escheria* which *Escherichia..Coli* belongs. In genus level, preoperative microbiome difference between clinical factors (**Table 2-4**) and microbiome difference between preoperative and postoperative in general is shown. (**Table 5**). *P*-value < 0.05 was considered as significant

difference. In species level, preoperative microbiome difference between clinical factors (**Table 6-8**) and microbiome difference between preoperative and postoperative in general is shown. (**Table 9**). As the number of species compared was too large, only top 30 were tabulated in general. p-value < 0.05 was considered as significant difference.

(A)



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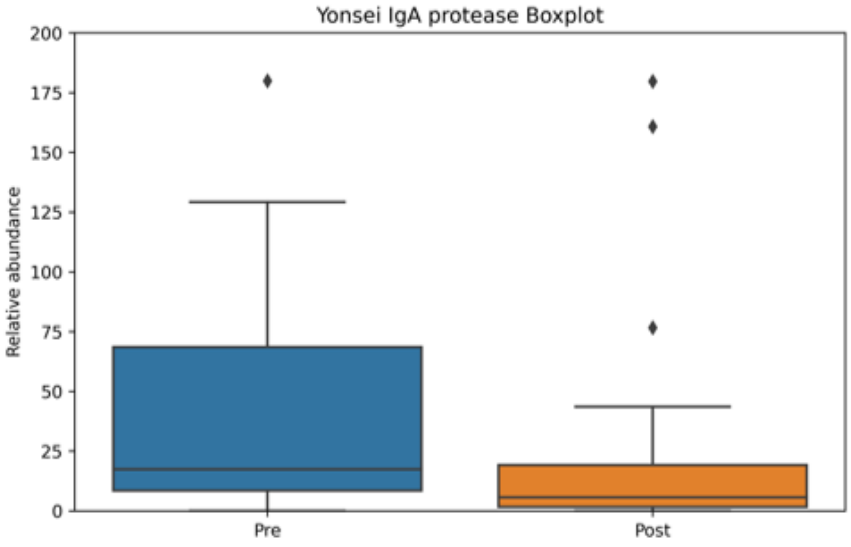

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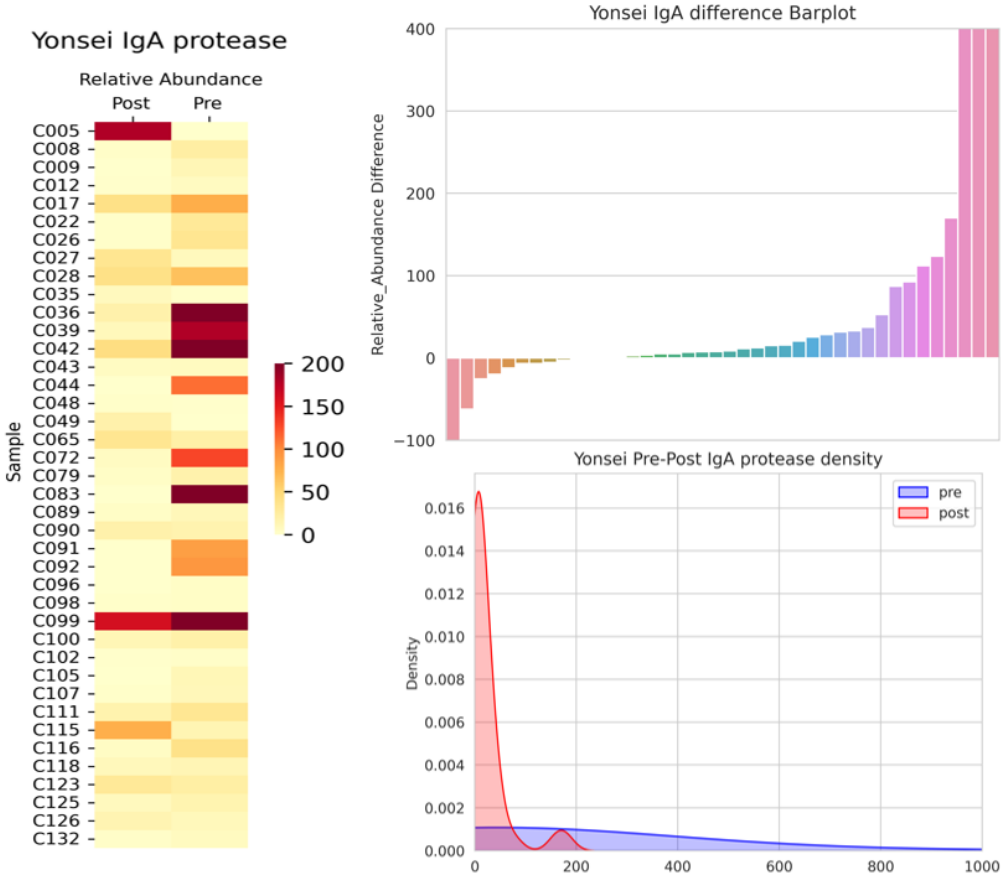

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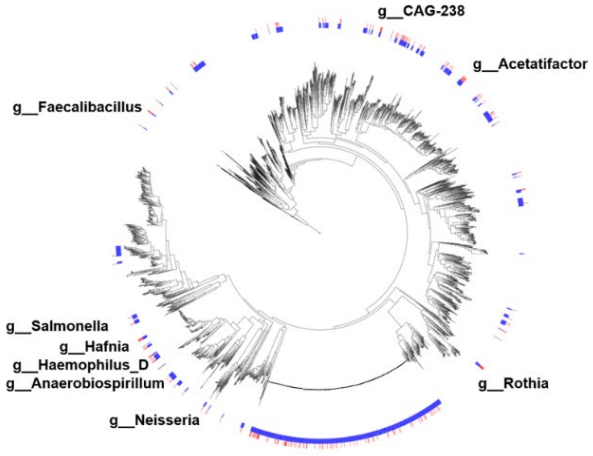

Genus	Average IgA protease gene copy number
g__Escherichia	10.02716641
g__Rothia	5.861111111
g__Aggregatibacter	5
g__Faecalibacillus	3.150232042
g__Anaerobiospirillum	3
g__HGM08974	2
g__UBA1390	1.75
g__Ruminococcus_C	1.579097843
g__Salmonella	1.5
g__Haemophilus	1.5
g__Lactococcus	1.486666667
g__Neisseria	1.307692308
g__Hafnia	1.25
g__UBA7160	1.214285714
g__UBA1227	1.166666667
g__CAG-632	1.009259259
g__Helicobacter_F	1

Figure 6 Identification of biomarkers for CRC-associated stool samples

(A) The volcano plots of KEGG Orthology (KO) differed in abundance between pre- and post-operative samples, (B) Box plots of IgA protease level between pre- and post-operative samples in human feces, (C) Density of IgA protease in each sample, (D) Distribution of genus abundances according to average copy number of IgA protease gene.

Table 2. Pre-operative microbiome difference by sex in the genus level

Bacteria (genus level)	Female (*n*=17)	Male (*n*=23)	*P* value
CAG.177	0.1 ± 0.2	0.4 ± 0.6	0.01
QALS01	0.0 ± 0.0	0.1 ± 0.2	0.041
Alistipes	5.5 ± 5.4	2.4 ± 3.0	0.045

Table 3. Pre-operative microbiome difference by tumor stage in the genus level

Bacteria (genus level)	Stages I-II (*n*=24)	Stages III-IV (*n*=16)	*P* value
BX7	0.0 ± 0.0	0.0 ± 0.0	0.007
Harryflintia	0.0 ± 0.0	0.0 ± 0.0	0.01
Cloacibacillus	0.0 ± 0.0	0.0 ± 0.0	0.034
Bilophila	0.3 ± 0.4	0.6 ± 0.5	0.045
HGM12998	0.0 ± 0.0	0.0 ± 0.0	0.045
UBA7067	0.0 ± 0.0	0.0 ± 0.0	0.046

Table 4. Pre-operative microbiome difference by tumor sideness in the genus level

Bacteria (genus level)	Left-sided (*n*=29)	Right-sided (*n*=11)	*P* value
Sutterella	0.4 ± 0.4	1.0 ± 0.8	0.039
Mitsuokella	0.1 ± 0.3	0.0 ± 0.0	0.044
Faecalibacillus	0.1 ± 0.2	0.2 ± 0.2	0.048

Table 5. Pre-operative and post-operative microbiome difference in the genus level

Bacteria (genus level)	Pre-operative feces (*n*=40)	Post-operative feces (*n*=40)	*P* value
Anaerotignum	0.2 ± 0.3	0.1 ± 0.1	0.001
Prevotella	14.9 ± 18.9	3.8 ± 9.1	0.001
Enterocloster	7.4 ± 7.3	14.1 ± 11.1	0.002
Lawsonibacter	0.1 ± 0.1	0.4 ± 0.6	0.003
Fusobacterium	0.1 ± 0.1	0.0 ± 0.1	0.003
Veillonella	1.7 ± 2.0	3.8 ± 4.2	0.006
Firm.11	0.1 ± 0.1	0.0 ± 0.0	0.008
Porphyromonas	0.7 ± 1.5	0.0 ± 0.2	0.009
CAG.110	1.0 ± 1.6	0.4 ± 0.6	0.013
Lactiplantibacilus	0.2 ± 0.3	0.8 ± 1.5	0.018
UBA7182	0.1 ± 0.1	0.1 ± 0.1	0.022
Agathobacter	2.4 ± 3.6	4.7 ± 5.5	0.034
CAG.460	0.1 ± 0.4	0.0 ± 0.1	0.044
Enterobacter	0.0 ± 0.1	0.2 ± 0.6	0.047

Table 6. Pre-operative microbiome difference by sex in the species level

Bacteria (species level)	Female (n=17)	Male (n=23)	*P* value
Butyricimonas.virosa.HRGMv2_0181	0.0119 ± 0.0219	0.0856 ± 0.1017	0.002
CAG.170.sp900549635.HRGMv2_2926	0.0291 ± 0.0460	0.1400 ± 0.1686	0.006
Desulfovibrio.sp900540515.HRGMv2_1733	0.0000 ± 0.0001	0.0150 ± 0.0249	0.009
Streptococcus.mitis_BB.HRGMv2_2537	0.0000 ± 0.0000	0.0002 ± 0.0002	0.018
Streptococcus.pseudopneumoniae O.HRGMv2 2534	0.0001 ± 0.0001	0.0004 ± 0.0005	0.021
Neisseria.macacae.HRGMv2_0602	0.0000 ± 0.0001	0.0002 ± 0.0003	0.022
CAG.83.sp000435975.HRGMv2_2816	0.0025 ± 0.0050	0.0487 ± 0.0953	0.03
UMGS1826.sp900555435.HRGMv2_2859	0.0019 ± 0.0037	0.0105 ± 0.0176	0.032
Unknown_0061.HRGMv2_0061	0.0001 ± 0.0001	0.0004 ± 0.0007	0.032
Enterocloster.sp000155435.HRGMv2_2275	0.0000 ± 0.0001	0.0008 ± 0.0016	0.036
OF09.33XD.sp003481995.HRGMv2_2187	0.0016 ± 0.0018	0.0006 ± 0.0005	0.036
Allisonella.histaminiformans.HRGMv2_3689	0.0050 ± 0.0144	0.0210 ± 0.0311	0.037
CAG.110.sp003525905.HRGMv2_2702	0.0466 ± 0.0583	0.1198 ± 0.1492	0.04
Parabacteroides.faecis.HRGMv2_0421	0.0000 ± 0.0000	0.0003 ± 0.0007	0.042
Collinsella.aerofaciens_G.HRGMv2_0889	0.0015 ± 0.0023	0.0090 ± 0.0168	0.043
Desulfovibrio.desulfuricans_A.HRGMv2_1731	0.0001 ± 0.0004	0.0009 ± 0.0017	0.045
Parabacteroides.timonensis.HRGMv2_0448	0.0000 ± 0.0000	0.0001 ± 0.0003	0.047
Abiotrophia.defectiva.HRGMv2_4356	0.0000 ± 0.0000	0.0001 ± 0.0001	0.049

Table 7. Pre-operative microbiome difference by tumor stage

Bacteria (species level)	Stages I-II (n=24)	Stages III-IV (n=16)	*P* value
Lachnospira.rogosae_A.HRGMv2_2061	0.6873 ± 0.7833	0.1207 ± 0.2527	0.003
BX7.sp014384765.HRGMv2_4513	0.0021 ± 0.0031	0.0001 ± 0.0003	0.007
Harryflintia.acetispora.HRGMv2_3067	0.0040 ± 0.0066	0.0003 ± 0.0007	0.01
Unknown_0890.HRGMv2_0890	0.0035 ± 0.0058	0.0002 ± 0.0004	0.011
Acutalibacter.sp900548545.HRGMv2_2908	0.0007 ± 0.0012	0.0001 ± 0.0002	0.013
Anaerotruncus.rubiinfantis.HRGMv2_3065	0.0030 ± 0.0037	0.0008 ± 0.0017	0.015
Collinsella.sp900546115.HRGMv2_1060	0.0001 ± 0.0002	0.0000 ± 0.0001	0.021
QAKL01.sp003343815.HRGMv2_3224	0.0208 ± 0.0377	0.0020 ± 0.0036	0.023
Unknown_0876.HRGMv2_0876	0.0017 ± 0.0027	0.0003 ± 0.0005	0.023
Anaerotruncus.sp014385085.HRGMv2_4517	0.0027 ± 0.0044	0.0005 ± 0.0011	0.025
AF33.28.sp003477885.HRGMv2_2364	0.0007 ± 0.0014	0.0001 ± 0.0003	0.03
Bilophila.wadsworthia.HRGMv2_1721	0.2954 ± 0.3196	0.5586 ± 0.4439	0.035
HGM13006.sp900756575.HRGMv2_3115	0.0004 ± 0.0006	0.0001 ± 0.0001	0.041
Haemophilus_D.sp900755445.HRGMv2_1753	0.0188 ± 0.0276	0.0061 ± 0.0078	0.042
Unknown_4408.HRGMv2_4408	0.0023 ± 0.0047	0.0002 ± 0.0006	0.042
UBA866.sp900543295.HRGMv2_3103	0.0040 ± 0.0054	0.0014 ± 0.0023	0.043
HGM12998.sp900756495.HRGMv2_4503	0.0035 ± 0.0061	0.0007 ± 0.0016	0.044
Lachnospira.sp900545725.HRGMv2_2106	0.0205 ± 0.0452	0.0009 ± 0.0030	0.044
Agathobacter.sp900548765.HRGMv2_2343	0.0001 ± 0.0001	0.0000 ± 0.0000	0.045
Ruthenibacterium.sp900546885.HRGMv2_2819	0.0104 ± 0.0234	0.0003 ± 0.0004	0.045
Unknown_4456.HRGMv2_4456	0.0002 ± 0.0004	0.0000 ± 0.0000	0.047
Unknown_3279.HRGMv2_3279	0.0001 ± 0.0003	0.0000 ± 0.0000	0.048

Table 8. Preoperative microbiome difference by tumor sideness in the species level

Bacteria (species level)	Left-sided (*n*=29)	Right-sided (*n*=11)	*P* value
Neisseria.macacae.HRGMv2_0602	0.0001 ± 0.0002	0.0000 ± 0.0000	0.003
Gemella.sanguinis.HRGMv2_4720	0.0004 ± 0.0007	0.0001 ± 0.0001	0.007
Streptococcus.mitis_BB.HRGMv2_2537	0.0001 ± 0.0002	0.0000 ± 0.0000	0.007
Lachnoanaerobaculum.orale.HRGMv2_2332	0.0001 ± 0.0001	0.0000 ± 0.0000	0.008
Unknown_2091.HRGMv2_2091	0.0069 ± 0.0111	0.0009 ± 0.0010	0.008
Harryflintia.acetispora.HRGMv2_3067	0.0034 ± 0.0062	0.0004 ± 0.0008	0.016
NSJ.63.sp014384805.HRGMv2_0817	0.0019 ± 0.0030	0.0004 ± 0.0005	0.019
CAG.103.sp000432375.HRGMv2_2709	0.0891 ± 0.1672	0.0117 ± 0.0157	0.02
Enterocloster.asparagiformis.HRGMv2_2357	0.0015 ± 0.0020	0.0005 ± 0.0008	0.02
Prevotella.hominis.HRGMv2_0281	0.0053 ± 0.0107	0.0004 ± 0.0006	0.02
Unknown_1432.HRGMv2_1432	0.0001 ± 0.0002	0.0000 ± 0.0000	0.022
Unknown_4620.HRGMv2_4620	0.0174 ± 0.0310	0.0031 ± 0.0049	0.022
Unknown_4528.HRGMv2_4528	0.0054 ± 0.0081	0.0016 ± 0.0021	0.023
SFEL01.sp004557245.HRGMv2_0681	0.2488 ± 0.4868	0.0276 ± 0.0760	0.024
Collinsella.sp003459245.HRGMv2_1609	0.0012 ± 0.0028	0.0000 ± 0.0000	0.025
UBA6984.sp003258725.HRGMv2_2017	0.0001 ± 0.0002	0.0000 ± 0.0000	0.031
Acutalibacter.sp900543555.HRGMv2_2880	0.0003 ± 0.0006	0.0001 ± 0.0001	0.032
Veillonella.tobetsuensis.HRGMv2_3727	0.0073 ± 0.0165	0.0004 ± 0.0012	0.033
Anaerofustis.stercorihominis.HRGMv2_1940	0.0002 ± 0.0005	0.0000 ± 0.0000	0.035
Dysosmobacter.welbionis.HRGMv2_2665	0.1610 ± 0.2112	0.0621 ± 0.0756	0.035
Gordonibacter.pamelaeae.HRGMv2_0859	0.0057 ± 0.0123	0.0006 ± 0.0010	0.035
Unknown_4534.HRGMv2_4534	0.0011 ± 0.0022	0.0001 ± 0.0004	0.035
Unknown_4316.HRGMv2_4316	0.0030 ± 0.0067	0.0002 ± 0.0006	0.037
Porphyromonas.uenonis.HRGMv2_0200	0.0008 ± 0.0020	0.0000 ± 0.0000	0.038
Lawsonibacter.sp000177015.HRGMv2_3112	0.0016 ± 0.0029	0.0004 ± 0.0006	0.039

Mitsuokella.jalaludinii.HRGMv2_3483	0.1474 ± 0.3509	0.0057 ± 0.0187	0.039
NSJ.61.sp003433845.HRGMv2_1907	0.0008 ± 0.0016	0.0001 ± 0.0002	0.039
Gemmiger.sp900540775.HRGMv2_2675	0.0066 ± 0.0123	0.0016 ± 0.0016	0.04
Collinsella.aerofaciens_J.HRGMv2_0933	0.0007 ± 0.0015	0.0001 ± 0.0003	0.041
CAG.568.sp000434395.HRGMv2_3437	0.0085 ± 0.0185	0.0011 ± 0.0017	0.042
Faecalibacterium.prausnitzii_D.HRGMv2_2772	0.6752 ± 0.7502	0.3125 ± 0.3396	0.043
Unknown_4075.HRGMv2_4075	0.0001 ± 0.0002	0.0000 ± 0.0000	0.043
Unknown_4468.HRGMv2_4468	0.0009 ± 0.0023	0.0000 ± 0.0001	0.044
Unknown_4540.HRGMv2_4540	0.0003 ± 0.0005	0.0000 ± 0.0001	0.045
Parabacteroides.sp900548175.HRGMv2_0341	0.0002 ± 0.0005	0.0000 ± 0.0000	0.046
Unknown_3184.HRGMv2_3184	0.0072 ± 0.0180	0.0002 ± 0.0005	0.046
Butyricicoccus.sp900547195.HRGMv2_3157	0.0004 ± 0.0010	0.0000 ± 0.0000	0.047
Aggregatibacter.segnis.HRGMv2_1846	0.0041 ± 0.0097	0.0003 ± 0.0007	0.048
Enterocloster.sp005845215.HRGMv2_2277	0.0009 ± 0.0022	0.0000 ± 0.0001	0.048
Rothia.mucilaginosa_B.HRGMv2_0069	0.0002 ± 0.0002	0.0000 ± 0.0001	0.048
Unknown_0876.HRGMv2_0876	0.0014 ± 0.0025	0.0003 ± 0.0008	0.048
Blautia_A.wexlerae.HRGMv2_2177	0.0005 ± 0.0010	0.0001 ± 0.0002	0.049

Table 9. Pre-operative and post-operative microbiome difference in the species level

Bacteria (species level)	Pre-operative feces (n=40)	Post-operative feces (n=40)	*P value*
Anaerotignum.faecicola.HRGMv2_2051	0.0742 ± 0.1163	0.2916 ± 0.3654	0.001
Sellimonas intestinalis.HRGMv2_2362	0.0006 ± 0.0015	0.0092 ± 0.0156	0.001
UBA7160.sp902363665.HRGMv2_2131	0.0008 ± 0.0018	0.0051 ± 0.0085	0.003
UBA9502.sp003506385.HRGMv2_2363	0.0019 ± 0.0062	0.0151 ± 0.0258	0.003
Mediterraneibacter.faecis.HRGMv2_2189	0.0564 ± 0.1177	0.2967 ± 0.4820	0.004
OF09.33XD.sp003481995.HRGMv2_2187	0.0028 ± 0.0037	0.0010 ± 0.0013	0.004
Unknown_3192.HRGMv2_3192	0.0019 ± 0.0058	0.0154 ± 0.0277	0.004
CAG.110.sp900549705.HRGMv2_2988	0.0014 ± 0.0048	0.0606 ± 0.1247	0.005
Lawsonibacter.asaccharolyticus.HRGMv2_2924	0.1444 ± 0.1884	0.0519 ± 0.0560	0.005
UMGS1312.sp900550625.HRGMv2_2825	0.0005 ± 0.0017	0.0157 ± 0.0323	0.005
Unknown_2371.HRGMv2_2371	0.0037 ± 0.0061	0.0116 ± 0.0161	0.005
Coprococcus_A.catus.HRGMv2_2085	0.0094 ± 0.0180	0.0264 ± 0.0331	0.006
Firm.11.sp900548145.HRGMv2_0735	0.0042 ± 0.0176	0.0580 ± 0.1162	0.006
Lawsonibacter.sp900066645.HRGMv2_3063	0.0609 ± 0.0905	0.0186 ± 0.0268	0.007
UMGS1375.sp900066615.HRGMv2_2053	0.0309 ± 0.0935	0.1751 ± 0.3065	0.007
Blautia_A.sp003477525.HRGMv2_4259	0.0005 ± 0.0015	0.0019 ± 0.0030	0.009
CAG.110.sp900540635.HRGMv2_2716	0.0019 ± 0.0054	0.1048 ± 0.2349	0.009
Prevotella.sp900557255.HRGMv2_0179	2.0792 ± 6.8464	8.7693 ± 14.3880	0.01
SFFH01.sp900548125.HRGMv2_0668	0.0039 ± 0.0089	0.0153 ± 0.0258	0.01
CAG.170.sp900545925.HRGMv2_2783	0.0074 ± 0.0151	0.0659 ± 0.1378	0.011
Dialister.sp900541485.HRGMv2_3746	0.0001 ± 0.0001	0.0003 ± 0.0006	0.013
ER4.sp000765235.HRGMv2_2744	0.6320 ± 1.1473	1.4059 ± 1.5424	0.013
CAG.127.sp900319515.HRGMv2_2056	0.7452 ± 1.4703	0.1447 ± 0.2689	0.015
Gemmiger.sp900540775.HRGMv2_2675	0.0009 ± 0.0020	0.0052 ± 0.0107	0.015

QAKL01.sp003343815.HRGMv2_3224	0.0009 ± 0.0032	0.0133 ± 0.0305	0.015
UBA644.sp900547165.HRGMv2_2837	0.0010 ± 0.0046	0.0102 ± 0.0223	0.015
UMGS1826.sp900555435.HRGMv2_2859	0.0011 ± 0.0029	0.0069 ± 0.0141	0.015
Anaerosaccharipilus.sp900066385.HRGMv2_2328	0.0049 ± 0.0096	0.0127 ± 0.0173	0.016
HGM13006.sp900757695.HRGMv2_2721	0.0010 ± 0.0023	0.0038 ± 0.0068	0.016
Lachnospira.sp000437735.HRGMv2_2105	1.0454 ± 2.1997	0.1635 ± 0.3145	0.016

IV. DISCUSSION

In this study, pre and postoperative stool microbiome of CRC patients showed its composition difference. There are few studies comparing preoperative and postoperative fecal microbiome from CRC patients. [14,15] Results from this study shows microbial taxonomic compositions and diversities of gut microbiota in post-surgery CRC patients were significantly different from pre-surgery CRC patients, which is similar with other study.[15] Cong Je et al reported that *Proteobacteria*, which is normally contained a minor portion in human gut microbiome has been increased in post-surgery, and in contrast, phylum *Fusobacteria* were more increased in pre-surgery.[15] Huo et al also reported that at the phylum level, the relative abundance of *Fusobacteria* at adjacent tumor sites is much higher in patients with CRC recurrence than that in patients without CRC recurrence.[16] Here, it was also able to confirm, as *Fusobacterium, Anaerotignum , Prevotella, and Porphyromonas* were ranked at the top in pre-surgery weigh. Preoperative difference seemed more important compared to postoperative difference, as they may more focused in recovering microbiome balance for bowel homeostasis. Clinical factors compared preoperative and postoperative microbiome in genus level, is shown in table 2-5.

Fusobacterium is the most famous known and studied microbiome, considered as CRC related facter. [17-19] Dysbiosis, with subsequent bacterial invasion, causes inflammation and inflammation causes cancer through a pro-inflammatory microenvironment that subsequently becomes a tumor microenvironment (TME), which downregulates the adaptive anti-tumor immune response and accelerates the CRC

progression. [20] Among several *Fusobacterium spp, F. nucleatum* is now considered a cancer-leading bacteria given its ability to stimulate oncogenic pathways through its proteins.

Anaerotignum and *Prevotella*, two strains were also identified as the strains with the greatest difference before and after surgery. *Anaerotignum* is assigned to Clostridium cluster XIVb belonging to the family *Lachnospiraceae*, proposed by Ueki et al. [21] It contains anaerobic, chemoorganotrophic, and fermentative bacteria that produce short chain fatty acids, including acetate, propionate, and butyrate.[22] Some studies report *Clostridium septicum, Clostridium difficile* are bacteria that are suspected to be related with colorectal cancer. [23,24] *Prevotella* seems overrepresented in adenocarcinoma compared to polyps, and also related to metastasis combined with *Fusobacterium nucleatum*.[25] However, *Prevotella* itself is still in controversy. Huh et al insisted that high abundance of *Prevotella* indicates lower risk of CRC progression and decease. [26] Moreover, *Porphyromonas* is also observed as oral bacteria related to periodontitis [27] and such genus are all known with pro-inflammatory, immunosuppressive, and tissue-invasive properties characters which may promote carcinogenesis. [28] Nagy et al. detected significantly higher levels of *Porphyromonas spp*. and *Fusobacterium spp*. in oral squamous cell carcinoma compared to adjacent healthy mucosa. [29]

In contrast, *Enterobacter, Lactiplantibacilus, Lawsonibacter, Enterocloster, Veillonella* were abundant in post-surgery. *Enterobacter* is gram negative, opportunistic, and important nosocomial pathogens that exist in many infections such as urinary tract

infections, bacteremia, pneumonia, meningitis. Dilsad et al noted that *Enterobacter* showed significantly increased cell viability and proliferation, while decreasing the apoptosis of the cell lines tested, thus could be a factor for initiation and progression for colon cancer. [30] *Lactiplantibacilus* strains are shown to inhibit colon cancer cell proliferation as function of its butyrogenic capability[31] and inhibit the growth of *Fusobacterium nucleatum.*[32] *Enterocloster* and *Lawsonibacter* are not well known for correlation with CRC yet, and *Veillonella* is known to be related to chemotherapeutic agent resistance.

By the difference of sideness, *Sutterella, Mitsuokella,* and *Faecalibacillus* showed abundance difference. *Sutterella* is gram-negative, anaerobic, non-spore forming bacteria found in human feces, and also main abundance in cecal content of rats which may be related to hind gut. [33] This study also reported *Sutterella* strain abundance in Rt.sided colon. *Mitsuokella* which is found more in Lt. sided, is more studied to depression or mood disorder. [34] Most of genus detected by tumor stage differentiation were not known well. *BX7, Harryflintia, Cloacibacillus* and *Bilophila* are some noted strains, however, its relation with CRC should be more studied.

Overall, well-known strains were discovered, however, *Sellimonas intestinalis* was one of the species that differed the most in this study. *Sellimonas intestinalis* was ranked at the high in post-surgery weigh and its prevalence has increased more than around 50%. This species was not well studied previously, however, it is known to help recovery after dysbiosis event.[35] In particular case of *Sellimonas intestinalis*, several genes associated with antibiotic resistance were found, so ability to carry antibiotic resistance

gene (ARG) could represent the basis for the survival of this species. The role of this species as a biomarker of homeostasis gut recovery, after presentation and restoration of homeostasis after dysbiosis could be expected.

This also helps explain changes in composition of the microbiome after surgery. This study shows definite alpha diversity difference, which means pre-surgery and post-surgery microbiome composition is different. Such bacterial difference may not be understandable, as surgeon only resects 10-20cm of bowel length. Under the assumption that strains that increase after surgery would have ARG, the result confirmed that high ARG species had less alpha diversity change compared to low ARG, in figure 6. Only the species with the most ARGS more than 4, which were the top 25% group confirmed by distribution plot were defined as high ARG species. Thus, for example, *Enterobacter* strains, even it is more related to CRC related one, were observed abundant in post-surgery as it has high antibiotic resistance.

By functional profiling, it appeared that postoperative stool microbiome of CRC patients revealed decreased Immunoglobin A (IgA) protease coding genes. IgA is an antibody that plays a crucial role in immune function of mucus membrane and it is reported that they participate in regulating gut commensal microbiome.[36,37] IgA protease is a serine endopeptidase to degrade IgA antibody. [36] The volcano plot shown above is the result of Kegg ortholog (KO) analysis, that IgA protease shows lowest p-value among pre abundant KO. To figure out if such species are pathogenic or not, copy number of its genus level was checked. Most of them were opportunistic pathogen such as *Escherichia, Rothia,*

Aggregatibacter. Therefore, these results showed that the number of pathogens were significantly reduced after surgery.

Unlike previous other study [15], to exclude bowel preparation effect and to compare stool within normalized life, stool was gathered after 3 to 6 months of post-surgery. This study confirmed that strains with ARG and decreased IgA coding genes remain for long time after surgery. Under the assumption that patient completely returned to normal diet, it appears that above strain can be studied as a factor related to the prognosis of colon cancer

V. CONCLUSION

Pre-surgery and post-surgery stool microbiome composition changes are significant. These changes may be due to strain characteristics such as antibiotic resistance genes and potential of IgA protease. Considering these factors, likewise species or genus will be more focused. With these clues, candidate for prognosis biomarker of colonic microbiome will more gained.

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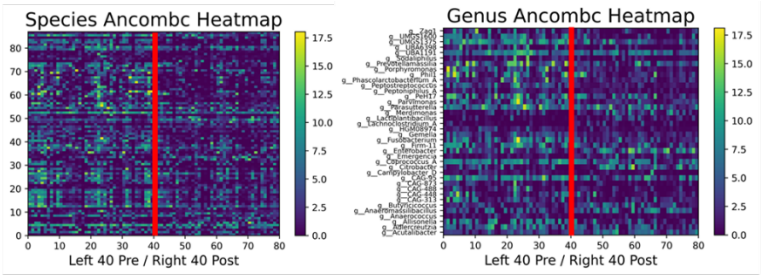
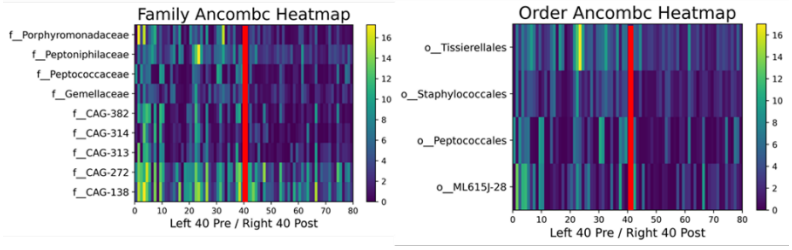
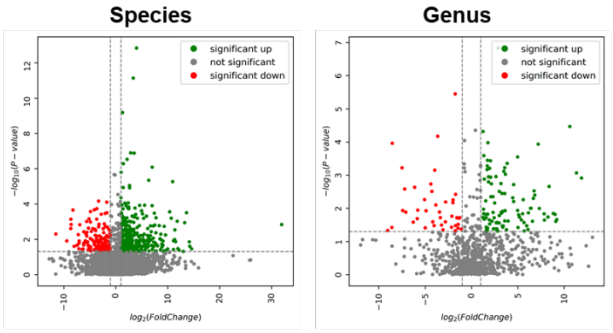
APPENDICES

(A)







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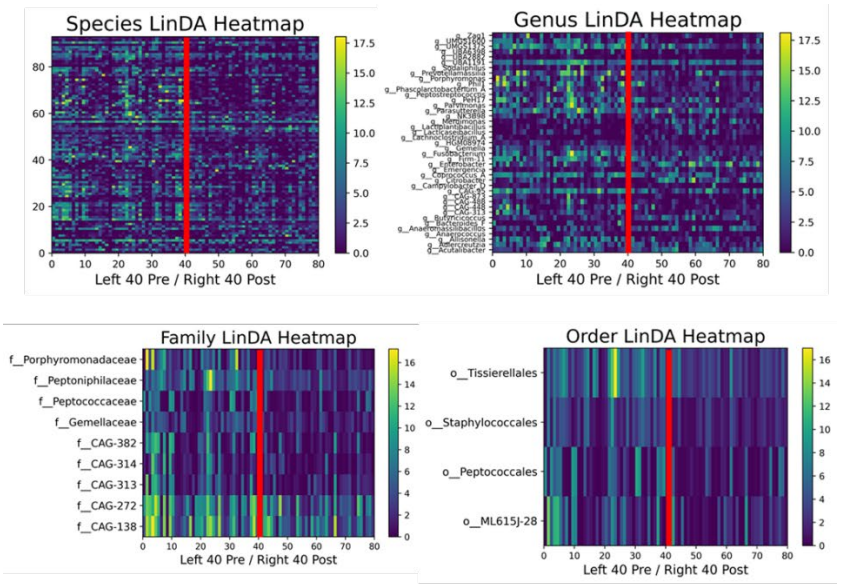
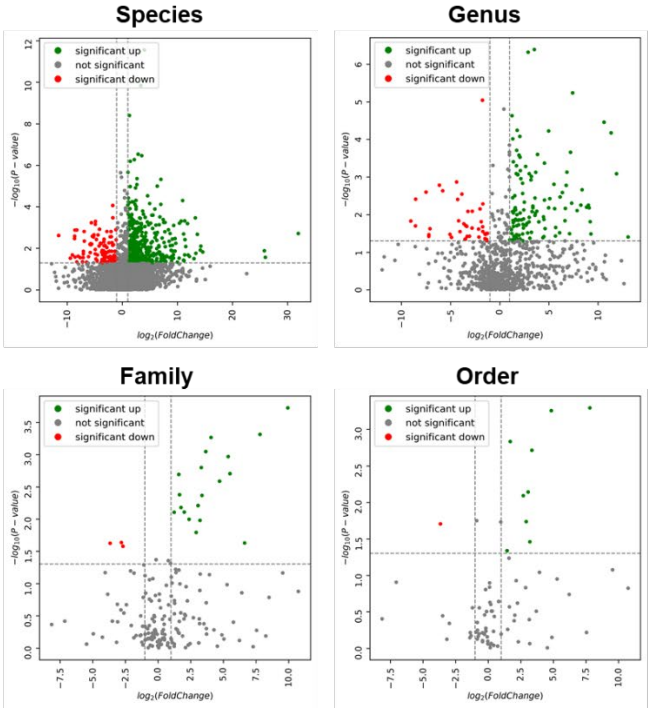
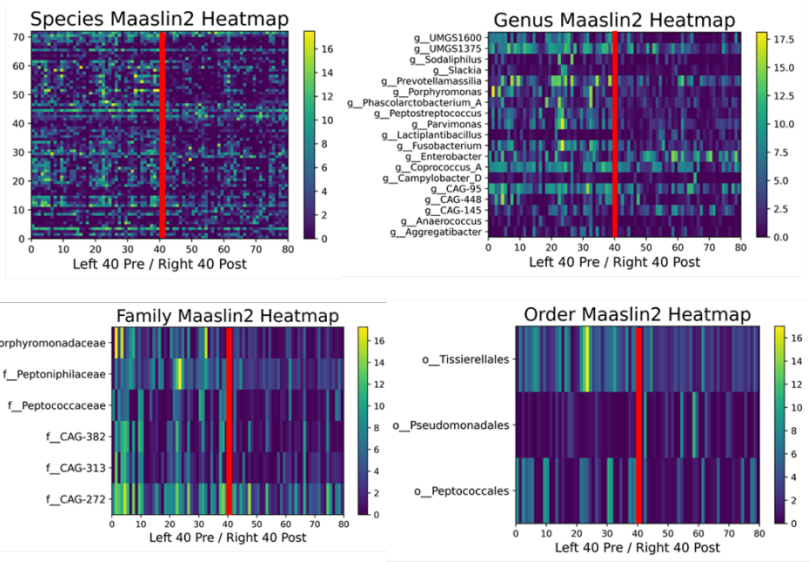
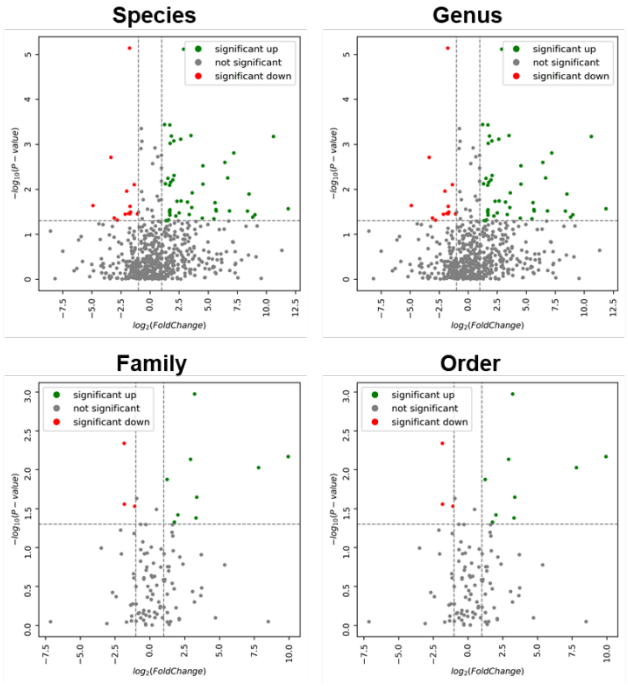




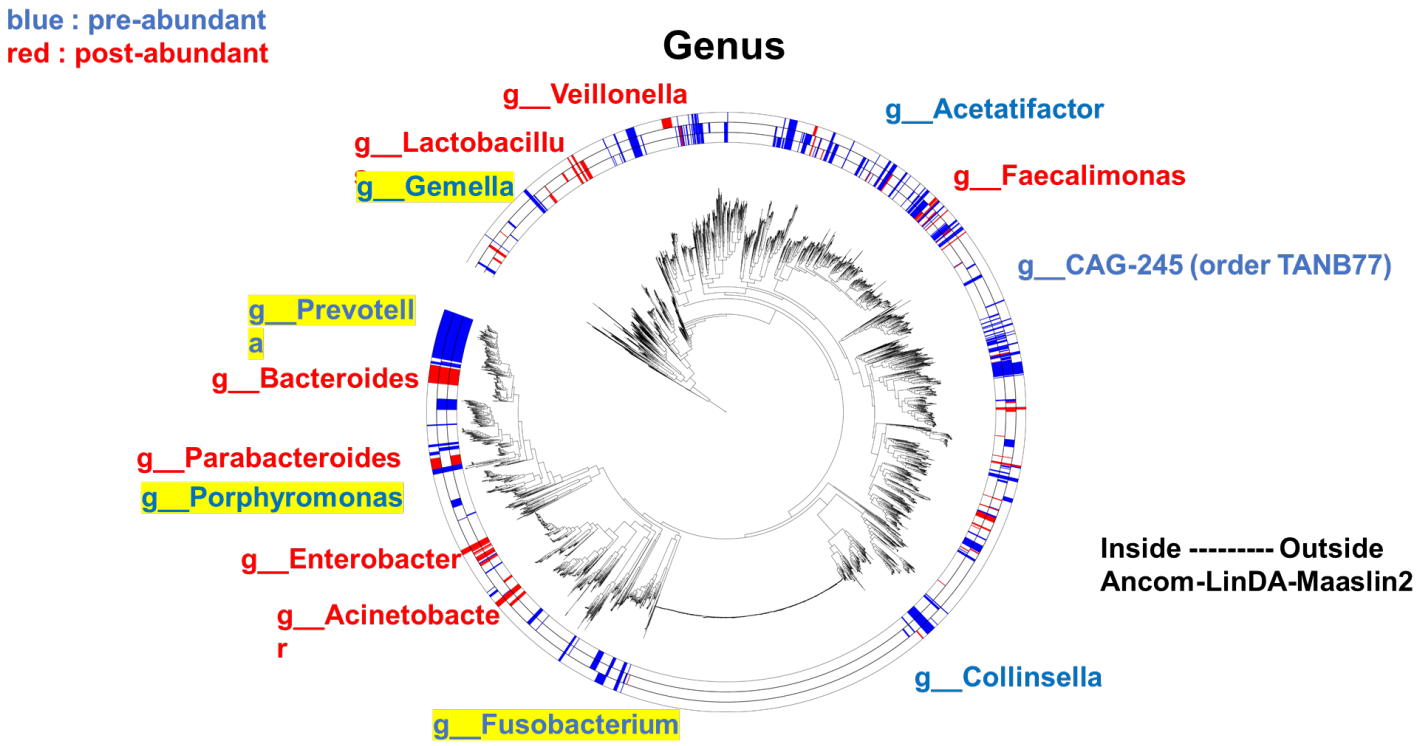
(C)



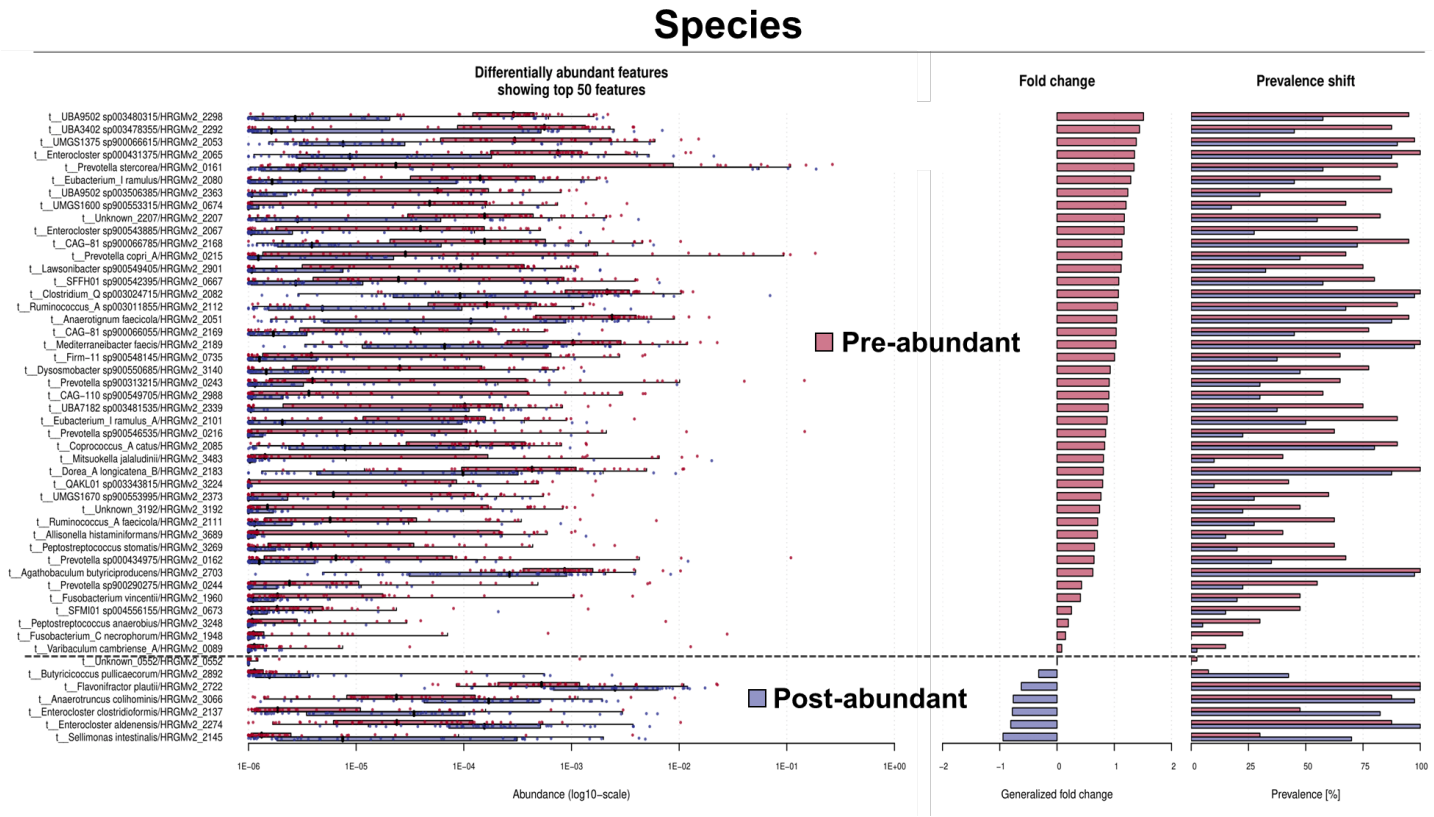


Supplementary Figure 1 Differential abundance (DA) analysis

(A) ANCOM- BC analysis, (B) LinDA analysis and (C) Maaslin2 analysis in the genus and species levels.



Supplementary Figure 2 cladogram of the hierarchy among discriminative taxa in the genus



Supplementary Figure 3 Pre-and post-operative difference in the species level

ABSTRACT (IN KOREAN)

대장암 환자의 수술 전후에 따른 장내 미생물 변화 비교 및 분석

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한윤대

배경: 대장암은 암 관련 사망의 주요 원인 중 하나이다. 최근에 대장암과 관련된 여러 위험 인자 중 장내 미생물은 대장암 주변 미세환경과 관련되어 주목받고 있다. 특정 장내 미생물이나 전형적으로 장내에 상주하는 미생물의 변화가 대장암 발병에 영향을 미칠 수 있다는 것이다. 그러나 아직 장내 미생물 군의 군집 조성 및 불균형에 대해 알려진 것이 많지는 않다. 이에 본 연구에서 수술 전후 대변을 통해 장내 미생물의 구성 변화를 분석하여, 이러한 변화가 대장암의 발달 및 진행에 어떠한 영향을 미칠 수 있는지 알아보고자 하였다.

방법: 수술 전후 대장암의 장내 미생물의 구성 변화를 확인하기 위해 총 40명의 대장암 환자에 있어서 수술 전과 수술 후 3-6개월 사이에 각각 대변을 수집하였다. 대변 샘플에서 장내 미생물 DNA를 추출하고 메타샷건 시퀀싱을 시행하였고, 미생물의 속 및 종 수준에서 이러한 분류학적 프로파일링을 수행한 후, 이를 토대로 조작분류단위 (Operational taxonomic units, OTUS), 알파 및 베타 다양성 분석, principal coordinate analysis (PCoA) 분석 및 differential abundance 분석으로 생물정보학적 분석을 시행하였다. 또한 immunoglobulin A (IgA) 단백효소분해제 항독소 여부를 확인하는 기능적 분석을 추가하였다.

결과: 속과 종 수준 모두에서 수술 후 알파 다양성과 OTUS가 감소하였으나 (p<0.05), 베타 다양성은 속 수준에서만 그 차이가 확연하였다. 특히, 항생제 내성 유전자가 적은 균주에서 많은 균주에 비해 알파 다양성의 변화가 많이

관찰되었다. PCoA 분석에서도 속과 종 수준 모두에서 수술 전후의 장내 미생물 군집 구성 변화가 관찰되었다. 속 수준에서 *Fusobacterium, Prevotella Peptostreptococcus* 등이 높게 검출되었고, 종 수준에서는 *Sellimonas intestinalis*가 수술 후 가장 많이 증가한 균주로 보고되었다. IgA 단백효소분해제 유전자의 copy 숫자가 높은 균주들은 대부분 *Escheria*나 *Rothia*, *Salmonella*, *Haemophilus*, *Helicobacter*처럼 병원체 균주로 알려진 것들이었다.
결론: 대장암의 수술 전후 장내 미생물의 구성 및 농도의 차이는 대장암과 연관성이 있다고 추정된다. 특히 *Fusobacterium*이나 *Prevotella* 등이 대장암과 관련이 있다고 보이며, 항생제 내성균 유전자나 IgA 단백효소분해제 포함 정도가 또한 군집 구성의 변화에 영향을 미치는 것으로 보인다. 앞으로 장내미생물의 변화에 대한 분석은 대장암 환자의 새로운 치료의 기점으로서 중요한 역할을 할 수 있을 것으로 보인다.

핵심되는 말: 마이크로바이옴, 장내미생물, 대장암