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The Gut Microbiome as a Promising Biomarker of Cancer Progression Among Female Never-smokers With Lung Adenocarcinoma

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Abstract

The gut microbiome plays an important role in the immune system and has attracted attention as a biomarker of diseases, including cancer. As such, in this study, we examined the relationship between the gut microbiome and lung cancer progression. Female never-smokers diagnosed with lung adenocarcinoma were consecutively enrolled between May 2018 and August 2019, and fecal samples were collected. Principal coordinate analyses were retrospectively performed using Bray-Curtis distance matrices to investigate the effects of clinical variables (age, body mass index, Tumor-Node-Metastasis stage, T category, N category, M category, primary tumor size, performance status, and EGFR mutation status) on the gut microbial community. A total of 37 patients were enrolled. T category and primary tumor size were significantly correlated with the gut microbial community (p=0.018 and 0.041, respectively). At the genus level, the relative abundance of *Faecalibacterium* was positively correlated with both T category and primary tumor size, whereas the relative abundances of *Fusicatenibacter* and *Bacteroides* were negatively correlated with these variables. This is the first study identifying the gut microbiome as a promising biomarker of lung cancer progression. Further elucidation of the role of the gut microbiome in lung cancer progression could facilitate development of new treatments for lung cancer.

Introduction

Lung cancer is the most prevalent cancer in the world, with 1.76 million deaths attributed to this disease in 2018, according to data from the World Health Organization. Unfortunately, as many lung cancer patients are diagnosed in the later stages of disease, when surgery or chemotherapy are less effective, the 5-year survival rate is only 16%.[1,2]

Although smoking is the most important cause of lung cancer, it is estimated that approximately 25% of lung cancer patients have never smoked.[3,4] Lung cancers in never-smokers are more common in women, and the histology is primarily adenocarcinoma.[4] Moreover, the prevalence of mutations in the epidermal growth factor receptor (EGFR) is reportedly high among female never-smokers with lung adenocarcinoma.[5] Although EGFR-mutation targeted therapy is often effective in these patients, the development of drug resistance is a serious problem.[6] The pathogenesis of lung cancer progression in never-smokers remains poorly understood, however, and thus should be elucidated in further studies.

The human microbiome plays an important role in the immune system, regulating various metabolic pathways under a wide range of conditions, including pulmonary diseases.[7] Several studies have suggested that the oral and lung microbiota contribute to the development of lung cancer.[8-10] However, the microbiota of the gut is the largest contributor to the human microbiome, weighing approximately 1.5 kg in total, and due to its prominence, the gut microbiome is attracting attention as a potential biomarker of diseases, including various cancers.[11-13] For example, Tanoue et al. reported that 11 specific bacterial strains in the feces of healthy human donors synergistically induce the accumulation of $CD8^+$ T cells,

enhancing the anti-tumor efficacy of immune checkpoint inhibitors in a mouse model.[14] In a study involving 100 patients (60 with advanced non-small cell lung cancer and 40 with renal cell carcinoma) who received anti–programmed cell death 1 immunotherapy, metagenomic analysis of patient stool samples at disease diagnosis revealed a correlation between the clinical response to immune checkpoint inhibitors and the relative abundance of *Akkermansia muciniphila*.[15] It is also gradually elucidated that changes in the composition of the gut microbial community are linked to the development of respiratory diseases such as asthma and cystic fibrosis, and this cross-talk between the gut and lung constitutes what is known as the "gut-lung axis".[13,16,17] Based on these data, it can be hypothesized that the gut microbiome impacts the progression of lung cancer.

Several recent studies comparing the gut microbiomes of lung cancer patients and healthy individuals also suggested that there is a relationship between the gut microbiome and lung cancer.[18,19] However, these studies were affected by two serious concerns. First, the patient group consisted of individuals with various histologic types of lung cancer. As the different histologic types of lung cancer develop via different mechanisms, lung cancer patients should be classified based on histology. Second, tobacco smoking affects the composition of the gut microbiome and is therefore an important potential confounding factor that must be considered when examining the relationship between the gut microbiome and lung cancer.[20] However, the abovementioned studies did not match the healthy individuals and lung cancer patients based on smoking history.

In the present study, we examined the relationship between the gut microbiome and cancer progression among female never-smokers with lung adenocarcinoma. In addition, we assessed the relationship between the gut microbiome and EGFR mutation status among these patients.

Results

Patient characteristics.

A total of 37 female never-smokers with histologically diagnosed lung adenocarcinoma were consecutively enrolled in this study between May 2018 and August 2019. The patients' clinical characteristics and laboratory data are summarized in Table 1. The average age and body mass index (BMI) were 69 years and 22.2 kg/m^2, respectively. The Japanese National Health Survey conducted in 2017 reported an average BMI of 23.1 kg/m^2 for females aged 60 to 74 years. Thus, the average BMI of the patients included in this study was similar to that of average Japanese women of the same age group. Most patients (n=27, 73%) had PS 0 disease, whereas 9 patients (24%) had PS 1, and only 1 patient (3%) had PS 2 disease. Therefore, PS was classified as 0 and ≥1 in the subsequent analysis. Table 1 also shows stratification of patients by TNM staging and EGFR mutation status. The size of the solid component of primary tumors is described as "primary tumor size" and also shown in Table 1. All of the patients' laboratory data were within normal ranges.

Table 1. Clinical characteristics and laboratory data for patients included in the study (n=37).

Variables	
Age (years), mean (SD)	69 (8)
BMI (kg/m^2), mean (SD)	22.2 (2.9)
PS 0/≥1, n (%)	27/10 (73%/27%)
TNM stage 0/Ⅰ/Ⅱ/Ⅲ/Ⅳ, n (%)	2/22/5/4/4 (5%/59%/14%/11%/11%)
T category is/1/2/3/4, n (%)	2/18/11/2/4 (5%/49%/30%/5%/11%)
N category 0/1/2/3, n (%)	27/3/4/3 (73%/8%/11%/8%)
M category 0/1, n (%)	33/4 (89%/11%)
Primary tumor size (mm), mean (SD)	26 (20)
EGFR mutation-positive, n (%)	20 (56%) (n=36)[a]
WBC (×10^3/μl), mean (SD)	5.9 (1.4)
Hb (g/dl), mean (SD)	12.7 (1.0)
Plt (×10^3/μl), mean (SD)	233 (57)
TP (g/dl), mean (SD)	7.1 (0.4)
Alb （g/dl）, mean (SD)	4.2 (0.3)
T-bil (mg/dl), mean (SD)	0.7 (0.2)
AST (U/l), mean (SD)	23 (11)
ALT (U/l), mean (SD)	20 (11)
LDH (U/l), mean (SD)	195 (42)
BUN (mg/dl), mean (SD)	17 (5)
Cr (mg/dl), mean (SD)	0.7 (0.2)
T-Cho (mg/dl), mean (SD)	212 (37)
HbA1c (%), mean (SD)	6.0 (0.6)
CRP (mg/dl), mean (SD)	0.2 (0.3)

[a] Number in parentheses indicates the number of patients with data available.

Alb, albumin; ALT, aspartate aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; Cr, creatinine; CRP, C-reactive protein; EGFR, epidermal growth factor receptor; Hb, hemoglobin; LDH, lactate dehydrogenase; Plt, platelet; SD, standard deviation; T-bil, total bilirubin; T-Cho, total cholesterol; TNM, Tumor-Node-Metastasis; PS, performance status; TP, total protein; WBC, white blood cell.

Effects of antibiotics on the gut microbiome.

We initially investigated whether the use of antibiotics before fecal sampling affected the gut microbiome of the patients included in the study. Although no patients reported using antibiotics within the 2-week period prior to fecal sampling, 4 patients used antibiotics within 3 months before sampling, and 3 of these patients used antibiotics within 1 month of sampling. A phylogenetic analysis found no similarities in the gut microbiome among patients who took antibiotics (Fig. 1). These data thus indicate that antibiotics use did not significantly affect the gut microbiome of patients enrolled in this study.

Effects of clinical variables on the gut microbial community.

PCoA was performed using Bray-Curtis distance matrices with the envfit function of the vegan R package to evaluate the effects of the clinical variables shown in Table 1 (age, BMI, PS, TNM stage, T category, N category, M category, primary tumor size, and EGFR mutation status) on the gut microbial community. This analysis identified T category and primary tumor size as significantly correlated with the composition of the gut microbiome. ($p=0.018$ and $p=0.041$, respectively) (Table 2). The PCoA plot is shown in Figure 2.

Table 2. Correlation of clinical variables with the gut microbiome community.

Variables	p value
Vectors	
Age	0.62
BMI	0.60
TNM stage	0.20
T category	0.018
N category	0.32
Primary tumor size	0.041
Factors	
PS: 0 vs ≥1	0.43
M category: 0 vs 1	0.12
EGFR mutation status: positive vs negative	0.11

BMI, body mass index; EGFR, epidermal growth factor receptor; PS, performance status; TNM, Tumor-Node-Metastasis.

Specific gut bacteria significantly associated with tumor progression.

A correlation analysis was performed to determine the strength of association between the dominant taxonomy (comprising ≥1% of the relative abundance of bacterial DNA sequences) and six clinical variables (vectors shown in Table 2). The results are shown in Figure 3. We particularly focused on clinical variables that significantly affected the gut microbial community (T category and primary tumor size) and examined specific gut bacteria associated with both of these variables. At the genus level, a significant positive correlation was observed between the relative abundance of *Faecalibacterium* and both T category (correlation coefficient, R=0.51, p=0.0013) and primary tumor size (R=0.37, p=0.024), whereas a significant negative correlation was observed between the relative abundances of *Fusicatenibacter* and *Bacteroides* and T category (R=−0.35, p=0.034 and R=−0.32, p=0.05, respectively) and primary tumor size (R=−0.36, p=0.029 and R=−0.41, p=0.012, respectively). Scatter diagrams depicting these relationships are shown in Figure 4. In addition, the relative abundances of *Faecalibacterium* and *Fusicatenibacter* were also correlated with TNM stage (R=0.31, p=0.06 and R=−0.34, p=0.039, respectively), although the former correlation was not statistically significant (Fig. 3).

Relationship between specific gut bacteria and EGFR mutation status.

Finally, we examined the association between the gut microbiome and EGFR mutation status. Although the PCoA indicated that EGFR mutation status did not have a statistically significant effect on gut microbial community (p=0.11) (Table 2), analysis of variance identified some bacteria at the genus level

as significantly associated with EGFR mutation status. Specifically, the relative abundances of *Bifidobacterium* and *Faecalibacterium* were significantly higher in EGFR mutation–negative patients than EGFR mutation–positive patients, whereas the relative abundance of *Blautia* was significantly lower in EGFR mutation–negative patients (Fig. 5).

Discussion

Research focusing on how the gut microbiome influences distant organs, such as the lungs, is increasing. The proposed “gut-lung axis” concept holds that dysbiosis of the gut microbiome or gut microbiota metabolites can modulate the immune system in the lungs and cause various respiratory diseases.[13,16,17] Indeed, metabolism of dietary fiber by the gut microbiota was shown to affect allergic airway diseases in mice.[21] Another study suggested that the gut microbiome affects the development of the microbial community of the respiratory tract in infants with cystic fibrosis.[22] However, there are few reports of studies investigating the relationship between the gut microbiome and lung cancer. Considerable recent attention has focused on the use of probiotics, living microorganisms that can improve the composition or function of the gut microbiota, in the context of treatments for various neoplasms, including lung cancer.[23,24] Probiotics are commonly found in yogurt, and an inverse association between yogurt intake and lung cancer risk was reported in a pooled analysis of 10 prospective cohorts involving 1,445,850 adults.[25] Based on these data, we considered it important to evaluate the relationship between the gut microbiome and lung cancer, as the resulting data could help to establish new lung cancer treatments targeting the gut microbiome.

In this study, we found that T category and primary tumor size are significantly associated with the gut microbial community among female never-smokers with lung adenocarcinoma. To our knowledge, this is the first study to identify the gut microbiome as a promising biomarker for lung cancer progression. Our analyses also revealed a positive correlation between the relative abundance of *Faecalibacterium* and tumor progression, whereas a negative correlation was found between the relative abundances of *Fusicatenibacter* and *Bacteroides* and tumor progression. Previous studies have demonstrated that some specific gut bacteria can affect immune cells in the tumor microenvironment[14,26]. This observation provides a potential clue for explaining the link between gut bacteria and lung cancer progression. For example, *Faecalibacterium prausnitzii* (sole known species within the genus *Faecalibacterium*), a common anaerobic bacterium usually representing more than 5% of the total gut bacterial population, upregulates regulatory T cells both *in vivo* and *in vitro*.[27,28] Generally, regulatory T cells suppress the activity of cytotoxic T cells, and it was reported that the number of tumor-infiltrating regulatory T cells is associated with worse recurrence-free survival in non-small cell lung cancer.[29] Based on these data, we speculate that *Faecalibacterium* plays a role in lung cancer progression by activating the function of regulatory T cells in the tumor microenvironment. Regarding *Bacteroides*, Vétizou et al reported that T-cell responses specific for *Bacteroides thetaiotaomicron* or *Bacteroides fragilis* were associated with the efficacy of cytotoxic T-lymphocyte-associated antigen 4 (a negative regulator of T-cell activation) blockade in mice and humans.[30] This result suggests that *Bacteroides* can upregulate T cells in the

tumor microenvironment and suppress tumor proliferation. It remains unclear whether *Fusicatenibacter* has an effect on immune cells because no detailed reports are available. Additional studies are needed to determine whether the specific bacteria identified in this study (*Faecalibacterium*, *Fusicatenibacter*, and *Bacteroides*) actually affect immune cells in the tumor microenvironment of lung cancer.

Our study has some clinical strengths, such as eliminating the confounding effect of smoking. Smoking is known to affect the gut microbiome and increase the numbers of *Bacteroides* and decrease those of *Firmicutes*.[20] In a previous study comparing the gut microbiomes of lung cancer patients and healthy individuals, lung cancer patients had higher levels of *Bacteroides* and lower levels of *Faecalibacterium* (of the phylum *Firmicutes*).[18] However, the percentage of ever-smokers in that study was higher among lung cancer patients than healthy controls (63.4% vs 43.9%). Thus, it is likely that the result of the study was influenced by the effect of smoking. Although changes in the gut microbiome due to smoking may be concerned with carcinogenesis, that potential link should be examined in future prospective studies. By contrast, our study completely excluded the possible confounding effect of smoking, because only never-smokers were included. Second, this is the first study to examine the relationship between EGFR mutation status and the gut microbiome. Regarding colorectal cancer, Burns et al previously reported that the tumor microenvironment microbial community is correlated with mutations in tumor DNA, and that this correlation can be used to predict mutated genes based on the microbiome.[31] Some bacteria identified in our study (*Bifidobacterium*, *Faecalibacterium*, and *Blautia*) were significantly correlated with EGFR mutation status. To the best of our knowledge, there are no reports describing the relationship between these bacteria and EGFR mutation status. With regard to the results of our study, we could not determine whether the changes in these bacteria occurred before or after the appearance of EGFR mutations. However, a previous study reported that *Enterococcus faecalis* can induce EGFR activation in human oral cancer cells via the production of a specific signaling molecule.[32] Thus, it is possible to hypothesize that the bacteria identified in this study are also associated in development of EGFR mutations and that they may play a crucial role in developing new treatments for lung cancers with EGFR mutations.

The potential limitations of the present study are as follows. First, the number of included patients was small. Thus, larger studies are needed to confirm the results of our study. Second, although this study provides initial insights into the relationship between the gut microbiome and lung cancer progression, it was not possible to determine whether alterations in the gut microbiome contribute to lung cancer progression or whether lung cancer progression changes the gut microbiome. To elucidate this cause-and-effect relationship, animal experiments or future longitudinal studies that incorporate repeatedly collected fecal samples will be necessary. Third, we used primary tumor size as a marker for cancer progression, but this parameter does not necessarily reflect the growth rate of the tumor. To evaluate cancer progression more accurately, for example, tumor volume doubling time is preferable. However, we could not measure tumor volume doubling time because of the study design.

In summary, our study identified the gut microbiome as a promising biomarker for cancer progression among female never-smokers with lung adenocarcinoma. By using probiotics or creating molecules that target microbial enzymes, we may expect to improve the efficacy of existing lung cancer therapies.

Methods

Study approval.

This retrospective study, performed at Kobe University Hospital, was approved by the Kobe University Hospital Clinical and Translational Research Center (approval no. B200044). Informed consent was obtained from all patients included in the study. All procedures performed were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Study population and data collection.

Female never-smokers histologically diagnosed with lung adenocarcinoma were consecutively enrolled in the study between May 2018 and August 2019. All patients included in the study lived in the same prefecture (Hyogo, Japan) and reported no unusual gastrointestinal symptoms within the 3-month period prior to enrollment. Fecal and blood samples were collected within 1 month before or after the diagnosis of lung cancer, before administration of any lung cancer treatment (surgery, chemotherapy, EGFR-mutation targeted therapy, or radiation).

Prior to fecal sampling, we determined whether antibiotics had been used by the patient. The eighth edition of the Tumor-Node-Metastasis (TNM) staging guideline for lung cancer was used to classify the extent of disease. Eastern Cooperative Oncology Group performance status (PS) and size of the solid component of the primary tumor were also assessed. EGFR mutation status was determined for all but one of the patients. Data from the enrolled patients were retrospectively analyzed in this study.

Fecal sample collection and bacterial DNA extraction.

Fecal samples were placed in 15-ml vials containing 3 ml of guanidine thiocyanate (GuSCN) solution (TechnoSuruga Laboratory Co., Ltd., Shizuoka, Japan). The samples were then mixed by vortexing and refrigerated at 4°C. Bacterial DNA was extracted from the samples in GuSCN solution according to a previously described bead-beating method.[33]

Amplification and sequencing of the 16S ribosomal RNA gene.

Bacterial DNA extracted from fecal samples was amplified by PCR. The V3-V4 regions of the bacterial 16S rRNA gene were amplified using the following primers: forward:

5’-TCGTCGGCAGCGTCAGATGTGTATAAGCGACAGCCTACGGGNGGCWGCA

G-3’, and reverse:

5’-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTAT

CTAATCC-3'. Detailed descriptions of the primer set and PCR conditions were reported elsewhere.[33] After addition of the sequencing adapters, the amplicons were sequenced using an Illumina MiSeq platform (Illumina Inc., CA, USA) according to the manufacturer's instructions.

Paired-end FASTQ files obtained from MiSeq were trimmed and merged prior to picking operational taxonomic units (OTUs). OTU classification and diversity analyses were performed using the QIIME pipeline (version 1.9.1).[34] All steps from FASTQ file trimming to analyses of gut microbiota diversity were performed automatically according to previously described methods.[35] OTUs were clustered against the SILVA 128 reference database at 97% similarity using the USEARCH algorithm.[36,37] The entire list of gut bacteria of each patient can be found in Supplementary Data file.

Data presentation and statistical analysis.

The resulting data were imported into R software (version 3.5.1) using BIOM files exported by the QIIME pipeline. α-Diversity indexes were calculated using the estimate_richness function in the "phyloseq" R package. β-Diversity indexes, calculated based on Bray-Curtis distances using genus-level data, were generated using the vegdist function in the "vegan" R package. Principal coordinate analysis (PCoAs) was carried out using the dudi.pco function in the "ade4" R package. Covariates of gut microbiome β-diversity variation were identified by calculating the associations between continuous or categorical clinical variables and genus-level community coordinates using the envfit function in the vegan R package. Correlations were analyzed using Spearman correlation analysis (cor function in "stats" R package). Heatmaps were created using the "superheat" R package, and other graphs were created using the R package "ggplot2". The statistical significance of differences was analyzed using JMP 9.0.2 software (SAS Institute Inc., Cary, NC, USA). Data are summarized as the mean (standard deviation) for continuous variables or number or percentage for categorical variables. In all cases, p values ≤ 0.05 were considered significant.

Data availability

The data supporting the results of the present study are available within the Supplementary Data file.

Declarations

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Author Contributions

T.O., T.N., T.Y., J.K. and Y.N. designed the study. T.O., T.N., J.P., K.H. and K.M. performed the statistical analysis and analyzed the data. T.O. and R.S. collected samples and obtained clinical data. All authors wrote and reviewed the manuscript.

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Figures

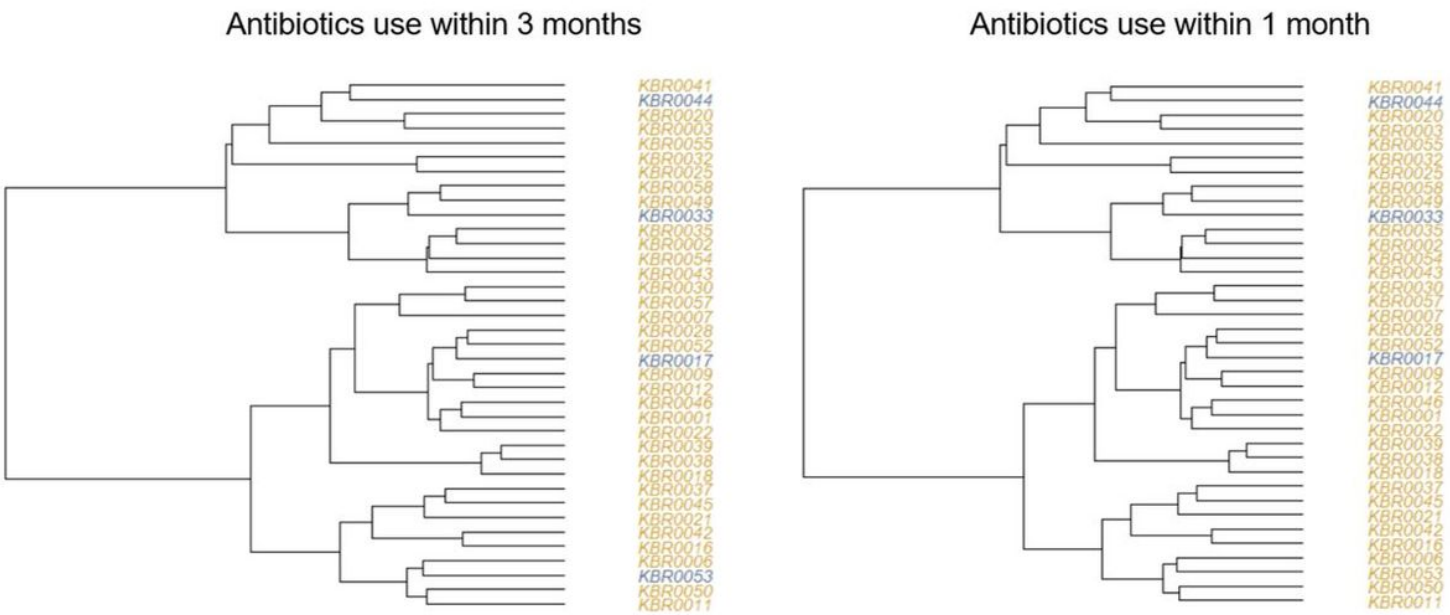
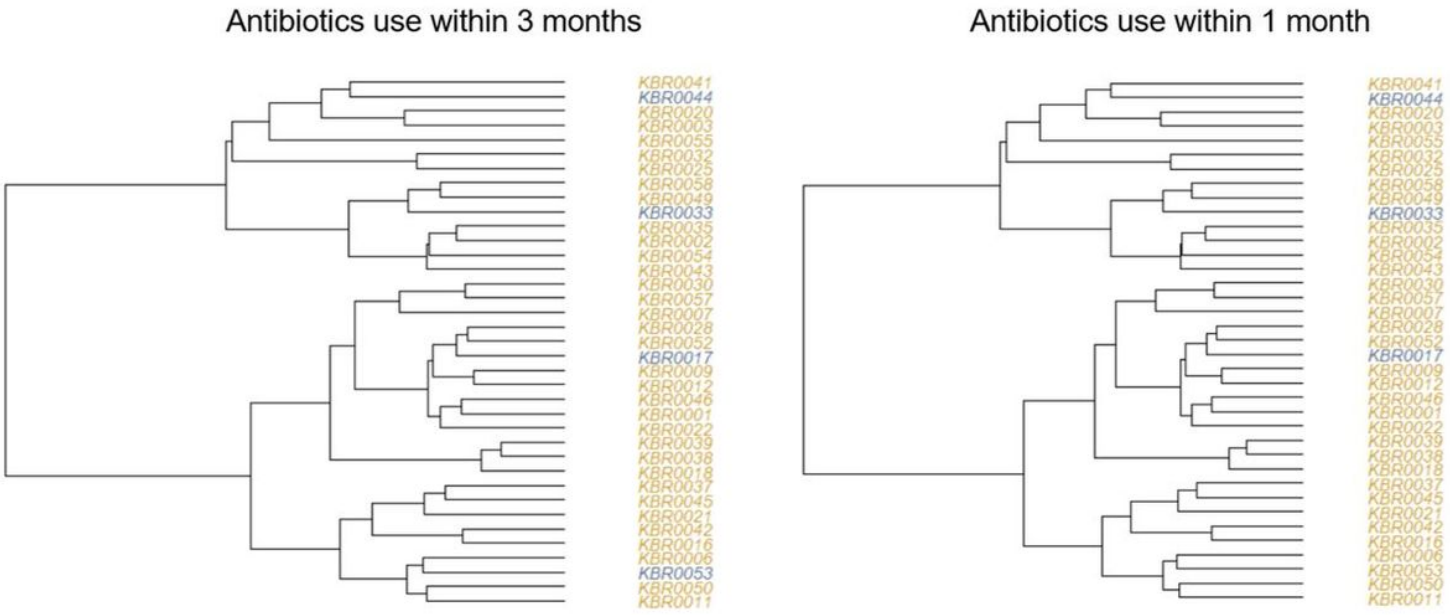


Figure 1

Phylogenetic analysis of the gut microbiome. Gray numbers are associated with patients who used antibiotics before fecal sampling within the indicated time period, and yellow numbers are associated with patients who did not use antibiotics within the indicated time period.



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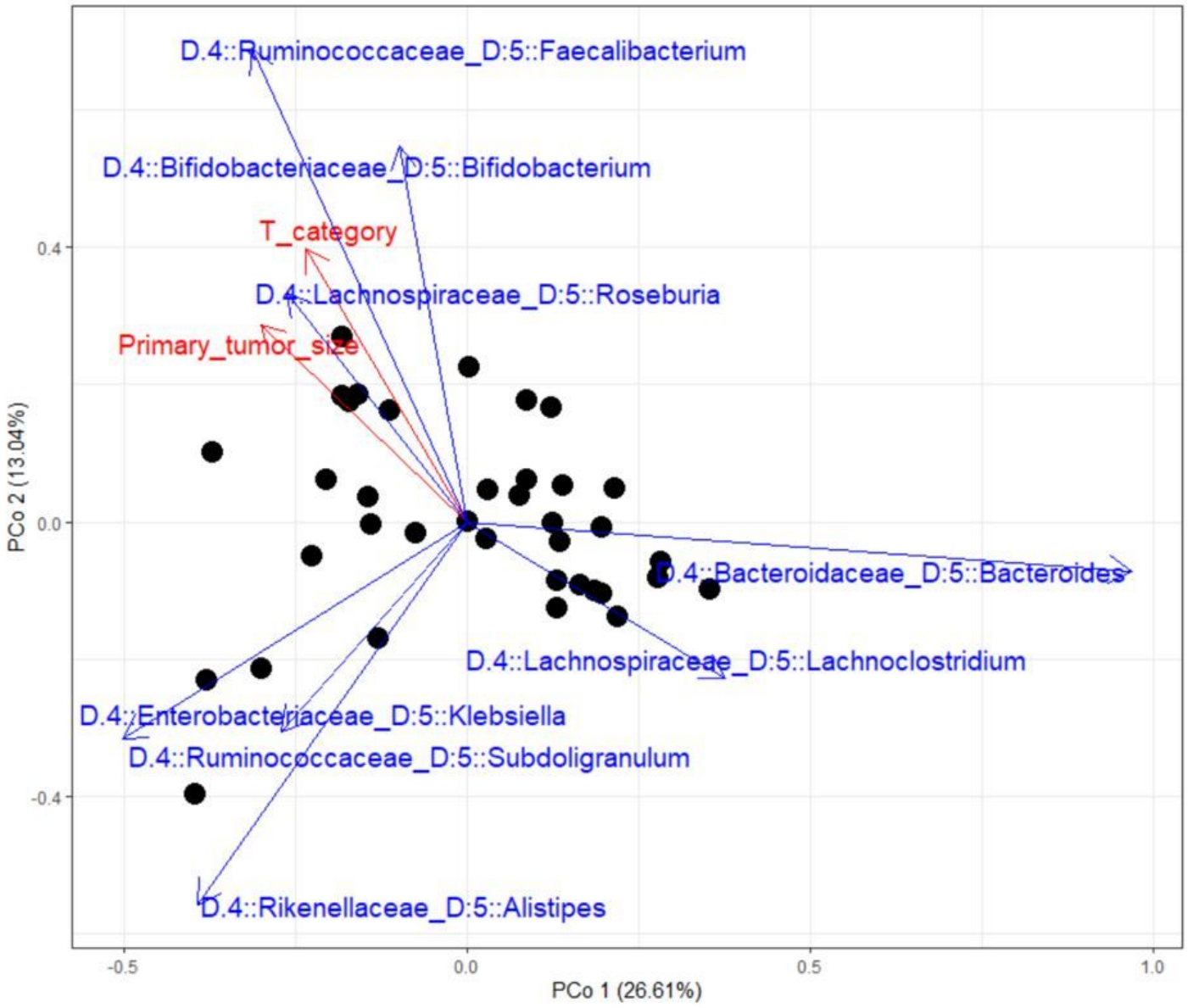
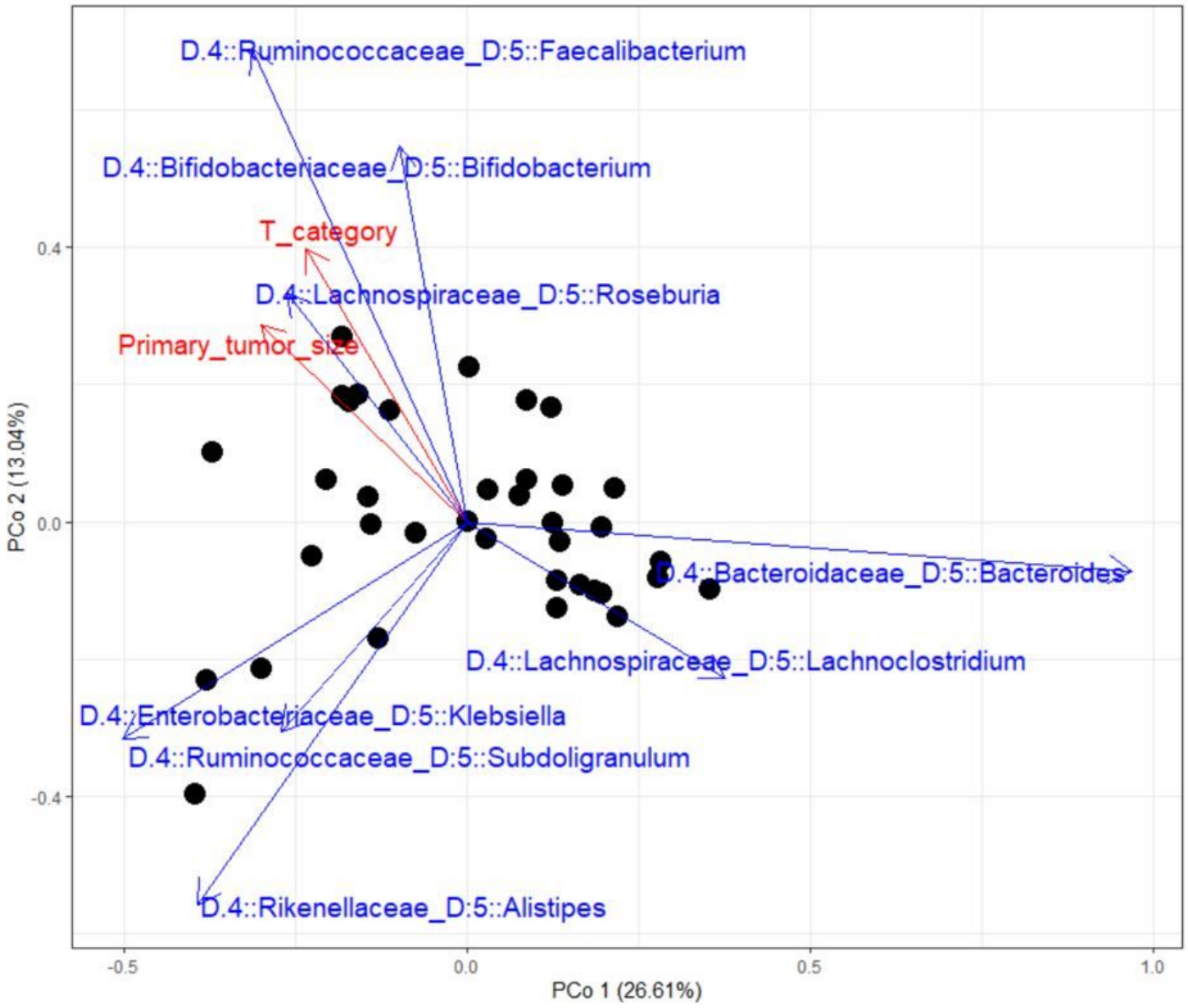


Figure 2

Principal coordinate (PCo) analysis plot based on Bray-Curtis distances. Each point represents an individual patient. Vectors in the figure indicate the direction in which each variable (T category, primary tumor size and the number of bacteria) increases. D4 and D5 in the figure represent family and genus level of bacteria, respectively. T category and primary tumor size were significantly correlated with gut microbial community.



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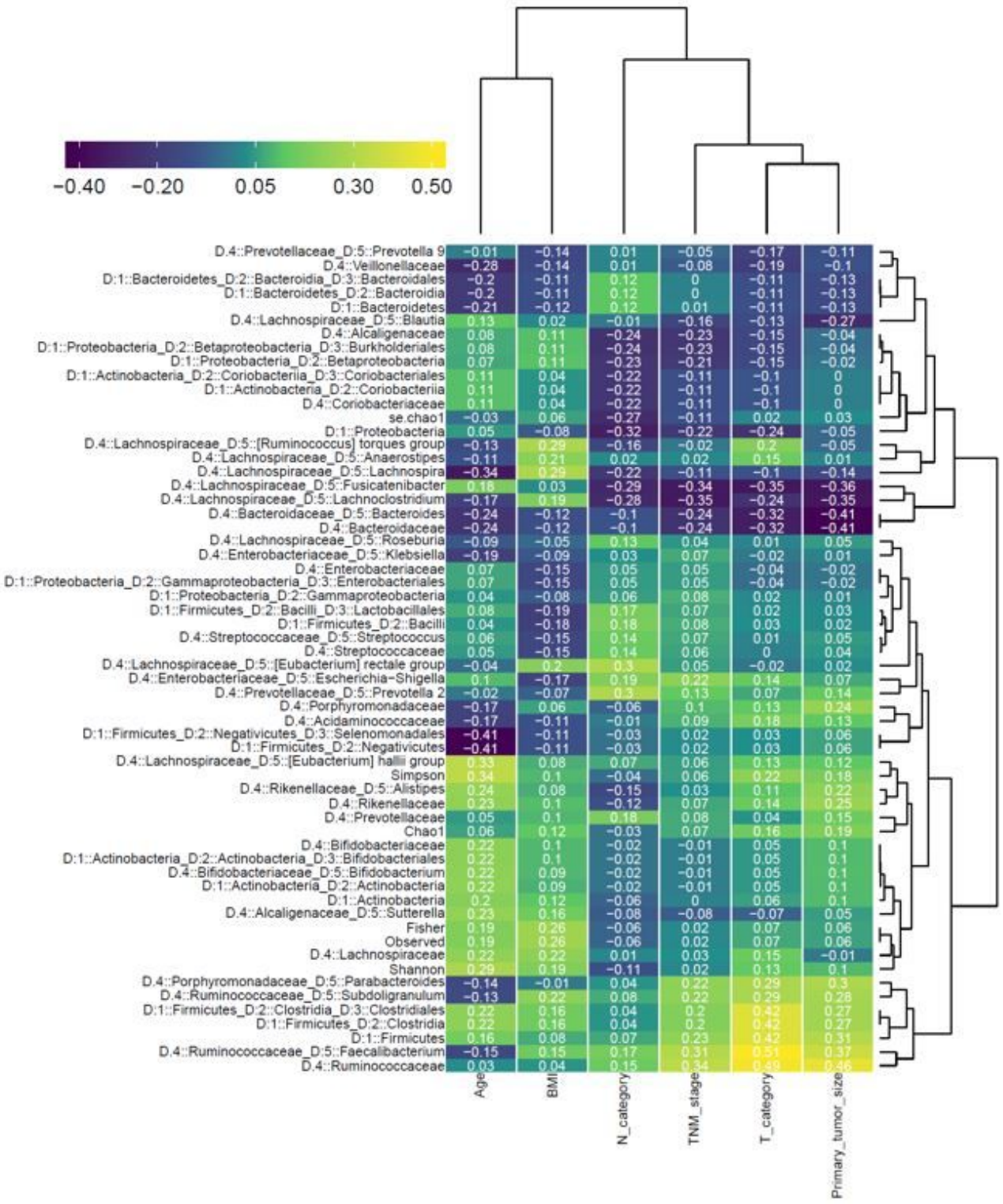
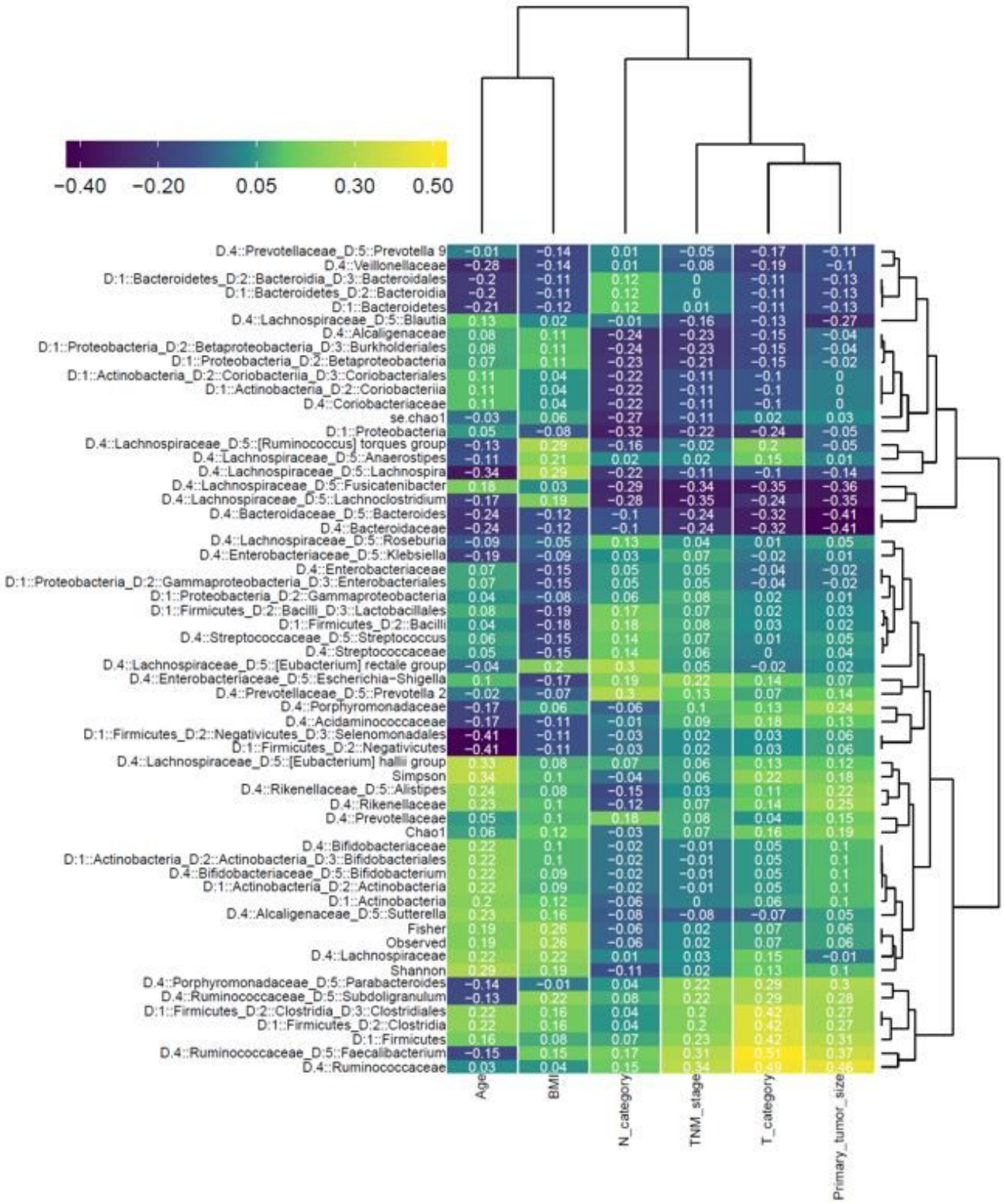


Figure 3

Correlation analysis to assess the strength of associations between the dominant taxonomy (comprising ≥1% of the relative abundance of bacterial DNA sequences) and each clinical variable. Numbers in the figure indicate the correlation coefficient. D1, D2, D3, D4, and D5 in the figure represent phylum, class, order, family, and genus level of bacteria, respectively.



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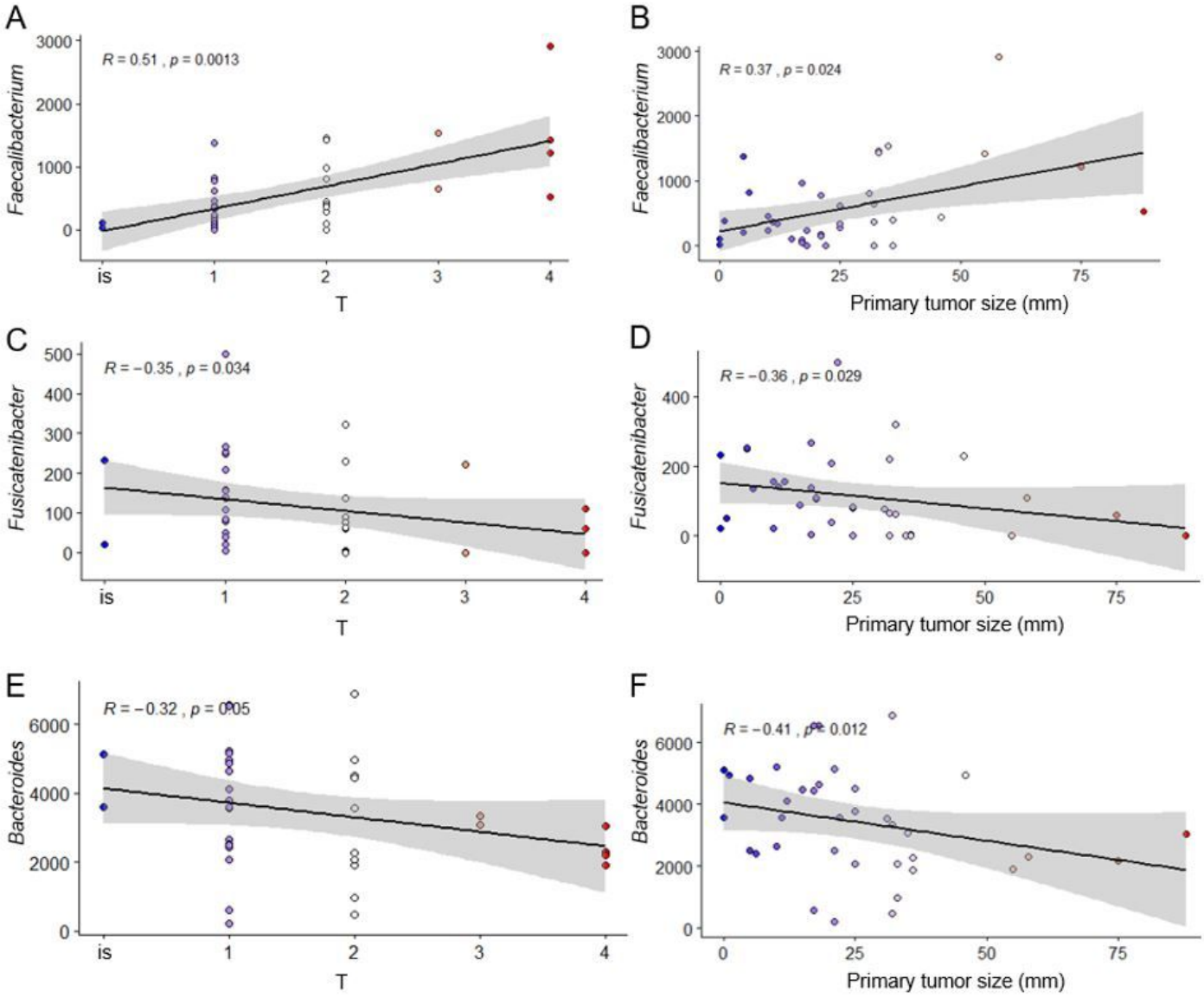
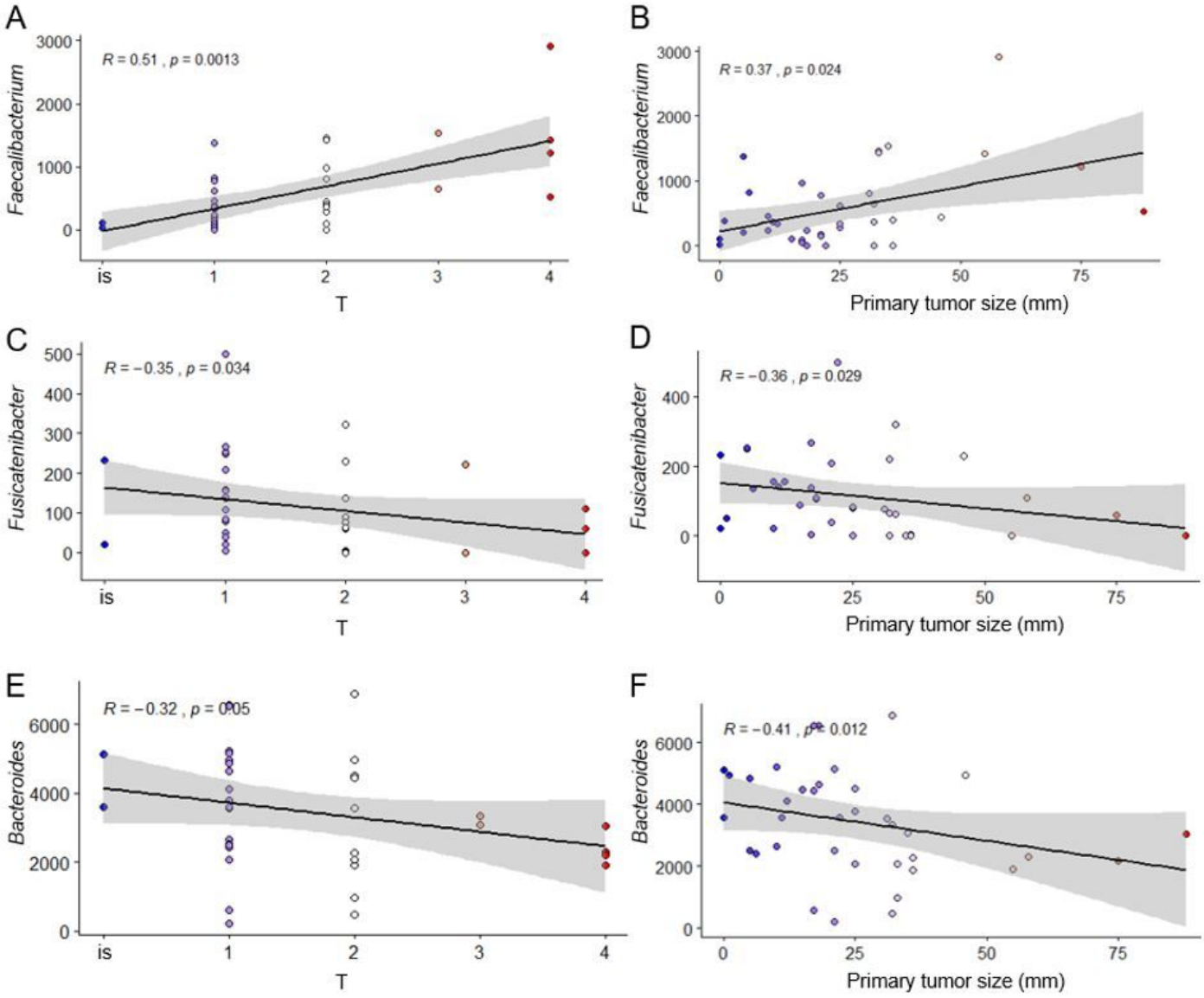


Figure 4

Scatter diagrams depicting the relationships between specific gut bacteria and T category or primary tumor size. T category and primary tumor size were correlated with the relative abundance of Faecalibacterium (A and B), Fusicatenibacter (C and D), and Bacteroides (E and F). Each point represents a single sample and is colored by T category and primary tumor size. The vertical axis shows the number of each bacterial genus among 104 total operational taxonomic units. R, correlation coefficient.



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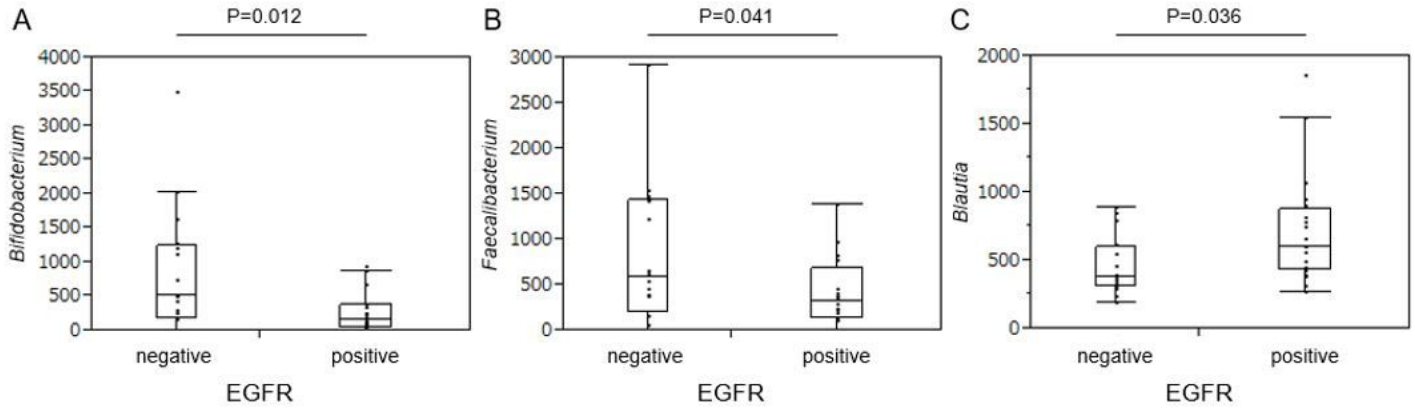
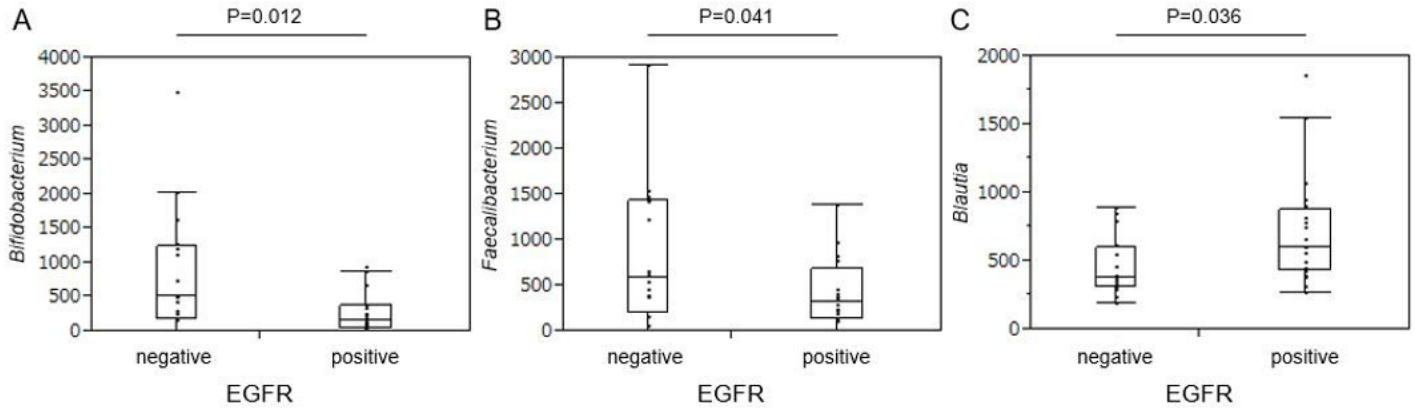


Figure 5

Boxplot showing the relative abundance of bacteria at the genus level that were significantly correlated with EGFR mutation status. (A) Bifidobacterium, (B) Faecalibacterium, and (C) Blautia. Vertical axis shows the number of bacteria from each genus among 104 total operational taxonomic units. Horizontal lines within boxes represent the median. The ends of the boxes represent the 75th and 25th quantiles. Whiskers extend from the ends of the boxes to the outermost data point that falls within 1.5-times the interquartile range. EGFR, epidermal growth factor receptor.



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Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryData.xlsx
- SupplementaryData.xlsx