The Gut Microbiome Associates with Immune Checkpoint Inhibition Outcomes in Patients with Advanced Non–Small Cell Lung Cancer

Taiki Hakozaki[1], Corentin Richard[2], Arielle Elkrief[2,3], Yukio Hosomi[1], Myriam Benlaïfaoui[2], Iris Mimpen[2], Safae Terrisse[4,5,6], Lisa Derosa[4,5,6], Laurence Zitvogel[4,5,6,7], Bertrand Routy[2,8], and Yusuke Okuma[1,9]

ABSTRACT

The gut microbiome (GM) plays an important role in shaping systemic immune responses and influences immune checkpoint inhibitor (ICI) efficacy. Antibiotics worsen clinical outcomes in patients receiving ICI. However, whether GM profiling and baseline antibiotic can be a biomarker of ICI efficacy in advanced non–small cell lung cancer (NSCLC) remains unknown. We prospectively collected baseline (pre-ICI) fecal samples and clinical data of 70 Japanese patients suffering from advanced NSCLC and treated them with anti–PD-1/PD-L1 antibodies as a first-line or treatment-refractory therapy. We performed 16S rRNA V3–V4 sequencing of gene amplicons of fecal samples, and bacteria diversity and differential abundance analysis was performed. The clinical endpoints were objective response rate (ORR), progression-free survival (PFS), overall survival (OS), and immune-related adverse events (irAE). ORR was 34%, and median PFS and OS were 5.2 and 16.2 months, respectively. Patients who received pre-ICI antibiotic had lower alpha diversity at baseline and underrepresentation of *Ruminococcaceae* UCG 13 and *Agathobacter*. When analyzing antibiotic-free patients, alpha diversity correlated with OS. In addition, *Ruminococcaceae* UCG 13 and *Agathobacter* were enriched in patients with favorable ORR and PFS >6 months. *Ruminococcaceae* UCG 13 was enriched in patients with OS >12 months. GM differences were observed between patients who experienced low- versus high-grade irAE. We demonstrated the negative influence of antibiotic on the GM composition and identified the bacteria repertoire in patients experiencing favorable responses to ICI.

See articles by Tomita et al., p. 1236, and Peng et al., p. 1251

Introduction

The therapeutic landscape of advanced non–small cell lung cancer (NSCLC) has been revolutionized by immune checkpoint inhibitors (ICI). Initial landmark trials compared single-agent anti–PD-1/PD-L1 mAbs to docetaxel in previously treated advanced NSCLC patients, which demonstrated improvements in overall survival (OS) with durable responses in the anti–PD-1/PD-L1 groups (1–4). As a result, the study of ICI in NSCLC rapidly expanded to first-line settings with or without platinum-doublet chemotherapy (5). Given the improved OS observed with first-line single-agent pembrolizumab in PD-L1–positive tumors (≥50%, using 22C3), this strategy is now the standard of care for this patient population (6).

Despite these unprecedented advances, disease progression with ICI therapy is often inevitable with primary resistance ranging from 35% to 44% (6–9). Predictive biomarkers of therapeutic success, based on PD-L1 expression and tumor mutational burden by genome profiling with next-generation sequencing, are inadequate given their limited sensitivity and specificity (10, 11). Finally, immune-related adverse events (irAE) remain an important therapeutic hurdle, leading to discontinuation of ICI in 7% of patients in clinical trials and up to 30% of patients in real-world settings (12).

Addressing these shortcomings in the immune-oncology landscape, the discovery that gut microbiome (GM) influences response to ICI, not only in NSCLC but also in melanoma and other tumors, illuminates the GM as a potential therapeutic target and biomarker of response. Indeed, in preclinical models, the efficacy of anti–CTLA-4 and PD-L1 antibodies require the presence of distinct *Bacteroides* species and *Bifidobacterium*, respectively (13, 14). Microbiome profiling in patients amenable to ICI identified that high bacterial GM diversity and specific commensals associate with $CD8^+$ T- and $CD4^+$ T-cell phenotypes and correlate with favorable response to ICI (15–18). Building on these findings, fecal microbial transplantation (FMT) from responder NSCLC patients into germ-free or antibiotic-treated specific pathogen–free mice restores the efficacy of ICI treatment in a $CD4^+$ $CXCR3^+$–dependent manner, whereas FMT from "nonresponder (NR)" patients abrogates ICI response (16).

Clinically, antibiotic use prior to the initiation of ICI associates with worse OS (19–21). The successful use of FMT to treat ICI-associated colitis reveals the potential role of the GM in abrogating irAE (22), demonstrating that the GM can be a therapeutic target to improve ICI and to dampen toxicity.

Notwithstanding these contributions to the understanding of local gut immunity on tumor immunosurveillance, the impact of antibiotics

[1]Department of Thoracic Oncology and Respiratory Medicine, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Bunkyo City, Tokyo, Japan. [2]University of Montreal Research Center (CRCHUM), Montreal, Quebec, Canada. [3]Segal Cancer Centre, Jewish General Hospital, Department of Oncology, Montreal, Quebec, Canada. [4]Gustave Roussy Cancer Campus (GRCC), Villejuif, France. [5]Institut National de la Santé Et de la Recherche Médicale (INSERM) U1015, Villejuif, France. [6]Univ. Paris-Sud, Université Paris-Saclay, Gustave Roussy, Villejuif, France. [7]Center of Clinical Investigations in Biotherapies of Cancer (CICBT) 1428, Villejuif, France. [8]Division of Medicine, Department of Hemato-Oncology, University of Montreal Healthcare Center, Montreal, Quebec, Canada. [9]Department of Thoracic Oncology, National Cancer Center Hospital, Chuo City, Tokyo, Japan.

Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

T. Hakozaki, C. Richard, A. Elkrief, B. Routy, and Y. Okuma contributed equally to this article.

Corresponding Authors: Yusuke Okuma, National Cancer Center Hospital, 5-1-1, Tsukiji, Chuo, Tokyo 104-0045, Japan. Phone: 313-3542-2511; E-mail: yokuma@ncc.go.jp; and Bertrand Routy, 900 Rue Saint Denis, Montreal, Quebec H2X 0A9, Canada. E-mail: bertrand.routy@umontreal.ca

Cancer Immunol Res 2020;8:1243–50

doi: 10.1158/2326-6066.CIR-20-0196

on the specific GM signature of patients with NSCLC remains to be fully defined. Whether GM profiling, at baseline or after antibiotic, could represent a biomarker of both response and irAE in advanced NSCLC during ICI therapy remains to be established. Here, we characterized the GM composition in a cohort of 70 patients with advanced NSCLC treated with single-agent anti–PD-1/PD-L1.

Materials and Methods

Patients

We prospectively collected fecal samples from 70 Japanese patients with advanced NSCLC receiving ICI between December 2017 and September 2019 who were treated at the Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital (Bunkyo City, Tokyo, Japan). Patients who fulfilled the following criteria were eligible for this study: (i) histologically or cytologically confirmed unresectable advanced (stage III or IV) or recurrent NSCLC and (ii) treatment with ICI (nivolumab, pembrolizumab, or atezolizumab) monotherapy at recommended dose either as the first-line or secondary therapy. Written informed consent was obtained from all patients. The study protocol was approved by the Ethics Committee of the Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital (Bunkyo City, Tokyo, Japan, approval number: #1744) and conducted in accordance with the tenets of the Declaration of Helsinki. The study was registered with the UMIN Clinical Trials Registry (ID: UMIN000021734).

Fecal samples at baseline (pre-ICI) were collected using a commercial sampling kit containing guanidine solution following the manufacturer's instructions (catalog no.: FS-0006, TechnoSuruga Laboratory Co., Ltd.). Fecal samples were immediately stored at 4°C and frozen at −80°C within 24 hours. Patient baseline characteristics including the use of antibiotic within 1 month before the first ICI injection were recorded.

16S rRNA gene sequence processing and analysis

Genomic DNA was extracted from fecal samples using NucleoSpin DNA Fecal Kit following the manufacturer's instructions (catalog no.: 740472.10, Macherey-Nagel GmbH & Co. KG) and immediately stored at −80°C. Isolated DNA was sent to TechnoSuruga Laboratory Co., Ltd. and analyzed using 16S rRNA gene sequencing to investigate the microbial composition in fecal samples. The V3–V4 hypervariable regions of the 16S rDNA were amplified using prokaryotic universal PCR primer pair Pro341F and Pro805R for the simultaneous analysis of bacteria and archaea (TechnoSuruga Laboratory Co., Ltd.). In addition to the V3–V4-specific priming regions, these primers were complementary to standard Illumina forward and reverse primers. To reduce the formation of spurious byproducts during the amplification process, the touchdown PCR methods for thermal cycling were used with a Rotor-Gene Q Quantitative Thermal Cycler (Qiagen). Sequencing was conducted using a paired-end, 2 × 250-bp cycle run on an Illumina MiSeq sequencing system and MiSeq Reagent Nano Kit version 2 (500 cycles) chemistry (23).

Gene sequence processing and analysis were performed using R v3.6.1 and GraphPad Prism v8.3.1. DADA2 (24) R package (25) v1.14.0 was used to generate exact amplicon sequence variants (ASV) of each sample from raw amplicon sequences. Sequences were corrected for Illumina amplicon sequence errors, dereplicated, chimera removed, and merged of paired-end reads with 260-bases for forward reads and 190-bases for reverse reads. The taxonomy assignment was performed against the SILVA reference database (v132; ref. 26). *Archea* and *Eukaryota* residual sequences were removed. Alpha diversity, defined as the number of distinguishable taxa, was analyzed at the genus level and computed with phyloseq R package (27) v1.30.0. The alpha diversity was estimated with different metrics: observed ASV, Shannon index, Inverse Simpson index, as well as weighted and unweighted Faith Phylogenetic Diversity (Faith pd, a phylogenetic index, and an analogue of taxon richness, and is expressed as the number of tree units which are found in a sample). Bray–Curtis distance (28) and weighted UniFrac distance (29) were used as beta diversity metrics (which show the difference in taxonomic abundance profiles from different samples) and visualized through NMDS method (30). Sequencing data were deposited at the SRA NCBI and are available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA606061/.

Statistical analysis

Statistical analysis was performed using R software v3.6.1. The Mann–Whitney *U* test was used to determine significant differences among the different groups using alpha diversity, which shows the diversity in each individual sample. Linear discriminant analysis (LDA) effect size (LEfSe; ref. 31) and DESeq2 (32) were used to perform differential abundances analysis at the genus level. The *P* values were corrected with the Benjamin–Hochberg procedure for the DEseq2 differential abundance analyses only. Both methods statistically enrich plausible bacterial strain to explain differences consistency and effect relevance by means of features using bioinformatics procedure. Multivariate analysis was performed by Cox proportional hazards model with a relative abundance of bacteria transformed with an arcsine-square root transform.

Clinical endpoints were OS (defined by the time of the first injection of ICI to the date of death from any cause), progression-free survival [PFS, defined by the time of the first injection of ICI to the first event (tumor progression or death from any cause)], objective response rate (ORR) based on RECIST v1.1 (33) criteria, and incidence and grade of irAE [graded by Common Terminology Criteria for Adverse Events (CTCAE) v4.0; ref. 34]. Patients with no events were censored at the date of the last follow-up. Survival curves were estimated through the Kaplan–Meier method and compared with the log-rank test (35, 36). Patients were defined as responders (favorable ORR) if they achieved complete response, partial response, or continuous stable disease for more than 6 months. Otherwise patients were defined as NRs (unfavorable ORR). For irAE, the highest grade toxicities during each therapy were recorded. To determine the effect of antibiotic on these clinical endpoints, the same analyses were performed in eubiotic patients who did not receive antibiotic. All tests performed were two-tailed. A $P < 0.05$ was considered statistically significant

Results

Baseline patient characteristics

The baseline characteristics of the 70 patients with NSCLC on ICI included in the study are presented in Supplementary Table S1. The median age in this cohort was 70 and most patients had an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of ≤1. All patients received anti–PD-1/PD-L1 monotherapy with 50% of patients treated in the first-line setting. Median follow-up for this cohort was 9.7 months. Sixteen patients (23%) received antibiotic 1 month prior to ICI initiation.

Gut microbial composition for all patients stratified by survival

First, we measured the alpha diversity for all patients in the cohort and found no difference in diversity between patients with

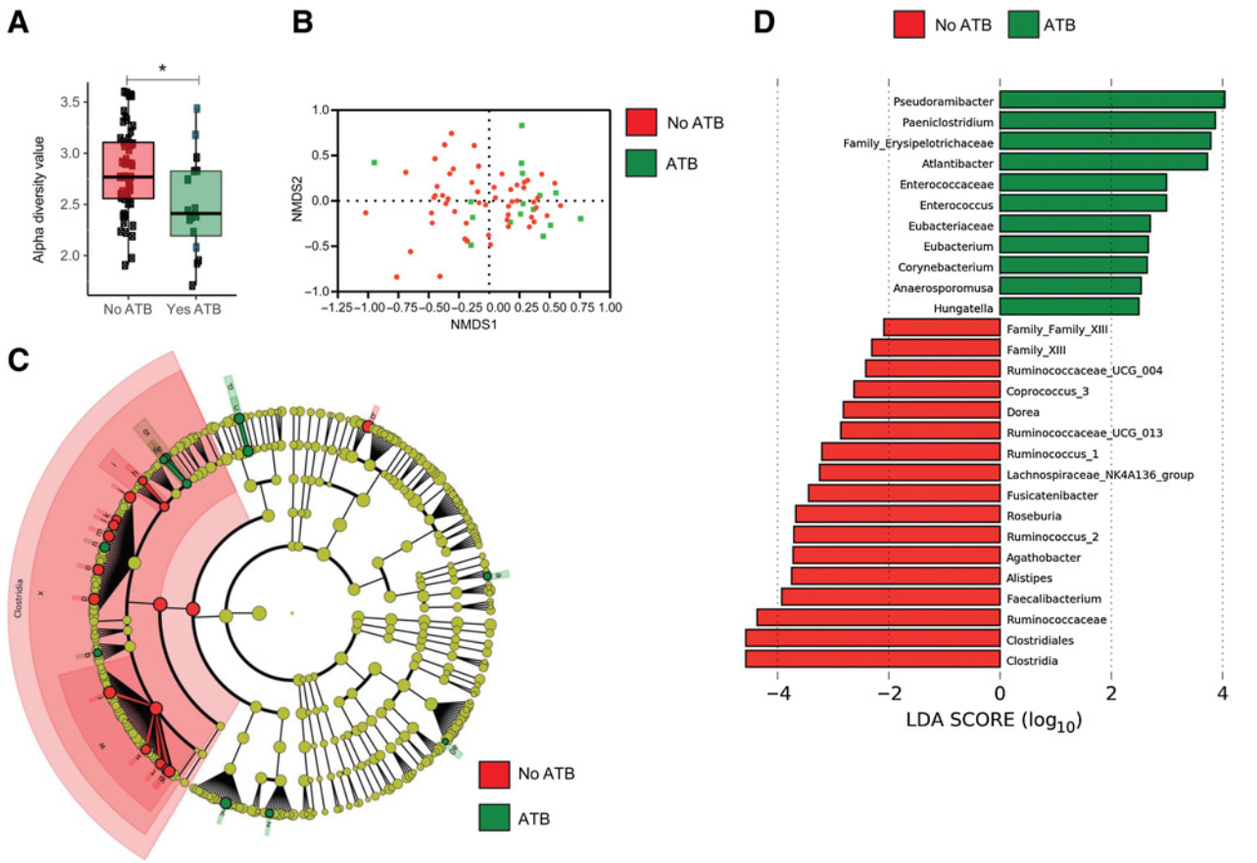

Figure 1.

GM composition for all ICI-treated patients with NSCLC stratified by antibiotic use. **A,** Alpha diversity for all patients stratified according to antibiotic use: antibiotic+ ($n = 16$) versus antibiotic free ($n = 54$) via Shannon index. The bold line represents the median. The bottom and top hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The top whisker extends from the hinge to the largest value no further than 1.5 * interquartile range from the hinge. *, $P < 0.05$. **B,** Beta diversity for all patients stratified according to antibiotic use. Note that all findings for beta diversity are statistically significant. **C,** Taxonomic cladogram stratified according to antibiotic. Dot size is proportional to the abundance of the taxon. Letters correspond to the following taxa: (a) *Corynebacterium*, (b) *Alistipes*, (c) *Enterococcus*, (d) *Enterococcaceae*, (e) *Eubacterium*, (f) *Pseudoramibacter*, (g) *Eubacteriaceae*, (h) Family XIII, (i) Family XIII, (j) *Agathobacter*, (k) *Coprococcus* 3, (l) *Dorea*, (m) *Fusicatenibacter*, (n) *Hungatella*, (o) *Lachnospiraceae* NK4A136 group, (p) *Roseburia*, (q) *Paeniclostridium*, (r) *Faecalibacterium*, (s) *Ruminococcaceae* UCG 004, (t) *Ruminococcaceae* UCG 013, (u) *Ruminococcus* 1, (v) *Ruminococcus* 2, (w) *Ruminococcaceae*, (x) Clostridiales, (y) Family *Erysipelotrichaceae*, (z) *Anaerosporomusa*, and (a0) *Atlantibacter*. **D,** Differential abundance analysis using LEfSe stratified according to antibiotic use. Note that all findings reported on LEfSe are statistically significant. ATB, antibiotic.

OS >12 months and those with OS ≤12 months (Supplementary Fig. S1A). We then compared GM composition between groups according to clinical outcomes. In patients with PFS >6 months, certain bacteria were enriched, including *Ruminococcaceae* UCG 13 and *Agathobacter* (Supplementary Fig. S1B). In patients with OS >12 months, *Lachnospiraceae* UCG 001, a member of the *Clostridiales* order, was also overrepresented (Supplementary Fig. S1C).

GM composition for all patients stratified by antibiotic use

We examined the impact of antibiotic on GM composition. Antibiotic use associated with significantly decreased alpha diversity by both Shannon (**Fig. 1A**) and Inverse Simpson methods (Supplementary Fig. S2). Two objective clusters were also found when analyzing beta diversity for antibiotic compared with antibiotic-free groups (**Fig. 1B**). Patients who did not receive antibiotic had feces that were enriched with Clostridia, specifically *Ruminococcaceae* UCG 13, Clostridiales, and *Agathobacter* (**Fig. 1C**), whereas feces from patients who received antibiotic were enriched in *Hungatella* (**Fig. 1D**).

GM composition for antibiotic-free patients and association with outcomes

Because antibiotic use significantly altered GM composition, we characterized GM composition in eubiotic patients by excluding patients who received antibiotic. In the antibiotic-free group, decreased alpha diversity associated with shorter OS (**Fig. 2A**). Of note, no objective clusters in the beta diversity analysis were found with regard to different outcomes. *Ruminococcaceae* UCG 13 and *Agathobacter* were overrepresented in those individuals with favorable ORR (**Fig. 2B**) and PFS >6 months (**Fig. 2C**). *Ruminococcaceae* UCG 13 was enriched in patients with OS >12 months (**Fig. 2D**). In antibiotic-free patients, Clostridiales order was also enriched in patients with OS >12 months (**Fig. 2D**). In addition, using DESeq2 analysis, dominance in *Ruminococcaceae* UCG 13 was also observed in patients with

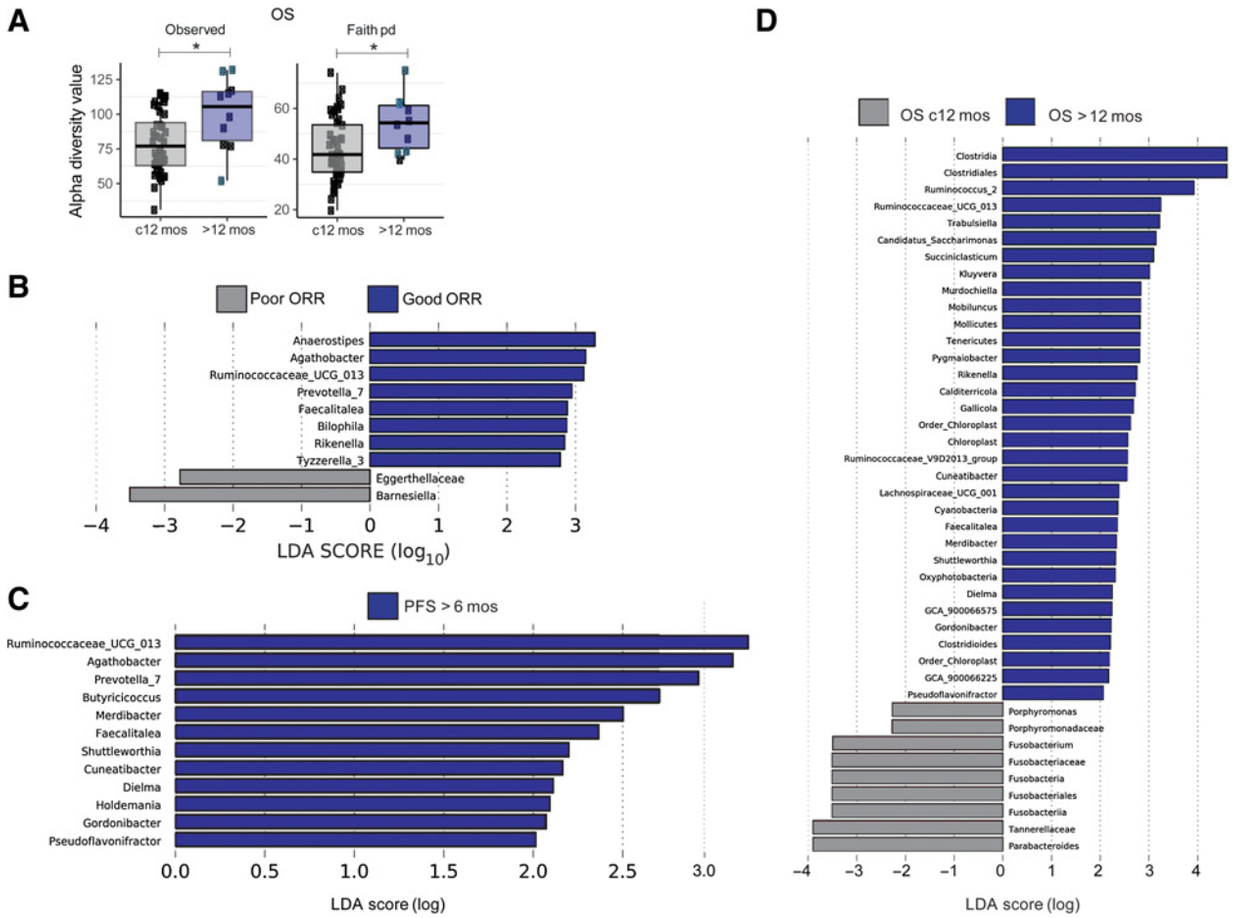

Figure 2.

GM composition for antibiotic-free ICI-treated patients with NSCLC and association with outcomes. **A,** Alpha diversity of patients who did not receive antibiotics ($n = 54$) stratified by OS by the Shannon index. The bold line represents the median. The bottom and top hinges correspond to the first and third quartiles (the 25th and 75th percentiles). The top whisker extends from the hinge to the largest value no further than 1.5 * interquartile range from the hinge. *, $P < 0.05$. **B,** Differential abundance analysis using LEfSe of antibiotic-free patients stratified by the objective response (OR); note that the presence of each organism on the LEfSe denotes statistical significance. **C,** Differential abundance analysis using LEfSe of antibiotic-free patients stratified by PFS. **D,** Differential abundance analysis (LEfSe) of antibiotic-free patients stratified by OS.

favorable ORR and PFS >6 months (Supplementary Fig. S3). Because *Ruminococcaceae* UCG 13 and *Agathobacter* were consistently enriched, we decided to focus on these particular members for subsequent analysis.

Hence, the Kaplan–Meier survival curves for patients with NSCLC whose GM contained or lacked *Ruminococcaceae* UCG 13 and *Agathobacter* significantly diverged. In antibiotic-free patients, the presence of *Ruminococcaceae* UCG 13 associated with longer OS [not reached vs. 4.4 months; HR, 0.24, 95% confidence interval (CI), 0.08–0.78; $P < 0.001$; **Fig. 3A**] and PFS ($P = 0.04$; Supplementary Fig. S4A). This was also true for *Agathobacter* in antibiotic-free patients for OS (not reached vs. 6.8 months; HR, 0.08; 95% CI, 0.02–0.3; $P < 0.0001$; **Fig. 3C**) and PFS ($P = 0.0001$; Supplementary Fig. S4B). Similarly, when analyzing all patients, the presence of *Ruminococcaceae* UCG 13 was associated with longer OS (not reached vs. 6.81 months; HR, 0.40; 95% CI, 0.16–0.96; $P = 0.03$; **Fig. 3B**). This association was also seen when analyzing *Agathobacter* for all patients for both OS (not reached vs. 5.5 months; HR, 0.13; 95% CI, 0.05–0.31; $P < 0.0001$) and PFS ($P = 0.0002$; **Fig. 3D**; Supplementary Fig. S4C). We then performed a multivariate analysis of the effect of *Ruminococcaceae* UCG 13 on OS taking into account standard prognostic factors relevant for advanced NSCLC including ECOG PS, histology type, lines of treatment, stage, and PD-L1 expression. The multivariate analysis further supported the presence of *Ruminococcaceae* UCG 13 associated with improved OS ($P = 0.004$; Supplementary Fig. S5). We performed multivariate analysis for *Agathobacter* as well, however, there was no statistically significant difference, $P = 0.59$ (Supplementary Fig. S6).

GM composition and association with irAEs

Given the possible association between the GM and irAE, we analyzed the differences in GM composition between patients who experienced clinically relevant (≥grade 2) irAE compared with those with nonsevere irAE (grade 1 or absent). In antibiotic-free patients and all patients, there was no difference in the bacterial diversity between these groups (Supplementary Fig. S7A and S7B). However, *Lactobacillaceae* on LEfSe analysis and *Raoultella* on DESeq2 analysis

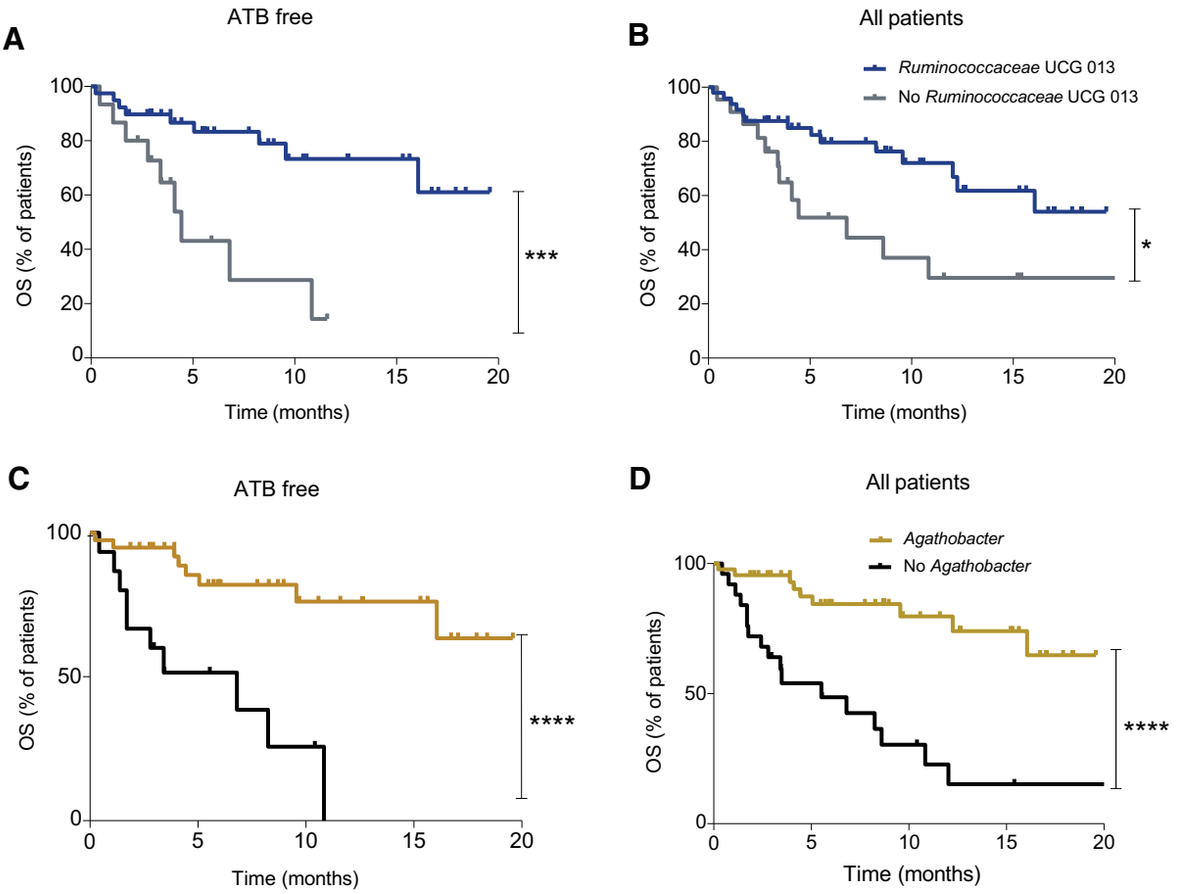

Figure 3.

OS stratified according to the presence or absence of *Ruminococcaceae* UCG 13 and *Agathobacter* in ICI-treated patients with NSCLC. **A,** Kaplan–Meier estimates of OS in antibiotic-free patients ($n = 54$) stratified according to the presence or absence of *Ruminococcaceae* UCG 13 using the log-rank test. **B,** OS of all patients ($n = 70$) stratified according to the presence or absence of *Ruminococcaceae* UCG 13 using the log-rank test. **C,** Kaplan–Meier estimates of OS in antibiotic-free patients ($n = 54$) stratified according to the presence or absence of *Agathobacter* using the log-rank test. **D,** OS of all patients ($n = 70$) stratified according to the presence or absence of *Agathobacter* using the log-rank test. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$. ATB, antibiotic.

were enriched in feces of patients who did not experience severe irAE profiles (**Fig. 4A** and **B**). Although *Akkermansia* species was not associated with improved clinical outcomes in our cohort, it was associated with less severe irAE profile (**Fig. 4B**). *Lactobacillaceae* and *Raoultella* also associated with a less severe irAE profile when examining all patients by LEfSe and DESeq2, respectively. Despite its association with favorable outcome, *Agathobacter* was associated with more severe irAE profile (Supplementary Fig. S8A and S8B).

Altogether, we demonstrated the negative influence of antibiotic on GM composition and identified a differential bacteria repertoire in patients experiencing favorable clinical outcomes (specifically, *Ruminococcaceae* UCG 13) or low grade irAE.

Discussion

In this prospective analysis of 70 patients with advanced NSCLC treated with monotherapy ICI, 16S rRNA sequencing revealed that patients who received antibiotic had lower alpha diversity at baseline and underrepresentation of Clostridiales, *Ruminococcaceae* UCG 13, and *Agathobacter*. When analyzing antibiotic-free patients, lower alpha diversity was observed in patients with lower OS. In addition, *Ruminococcaceae* UCG 13 and *Agathobacter* were overrepresented in patients with favorable ORR and PFS >6 months. *Ruminococcaceae* UCG 13 was enriched in those with OS >12 months. Clostridiales order was also enriched in patients with OS >12 months. Compositional GM differences were also observed between the patients who experienced clinically significant (≥grade 2) irAE; *Lactobacillaceae* and *Raoultella* were enriched in patients who had less severe irAE profile, whereas *Agathobacter* associated with more severe irAE profile.

Our findings corroborate the key role of the GM diversity, defined by the abundance distribution of microorganisms colonizing the gut, in predicting beneficial responses to ICI. Indeed, several investigators profiling the microbiome across tumor histology, ICI type, and geographic distribution demonstrate the association between higher baseline diversity and favorable clinical outcomes (15–18, 37). Beyond diversity metrics, specific commensals associate with improved outcomes to ICI such as objective response and survival (38). Increases in both *Clostridiales* and *Ruminococcus* in 43 patients with advanced melanoma correlate with favorable response to anti–PD-1. When analyzing systemic immune responses in patients enriched with these

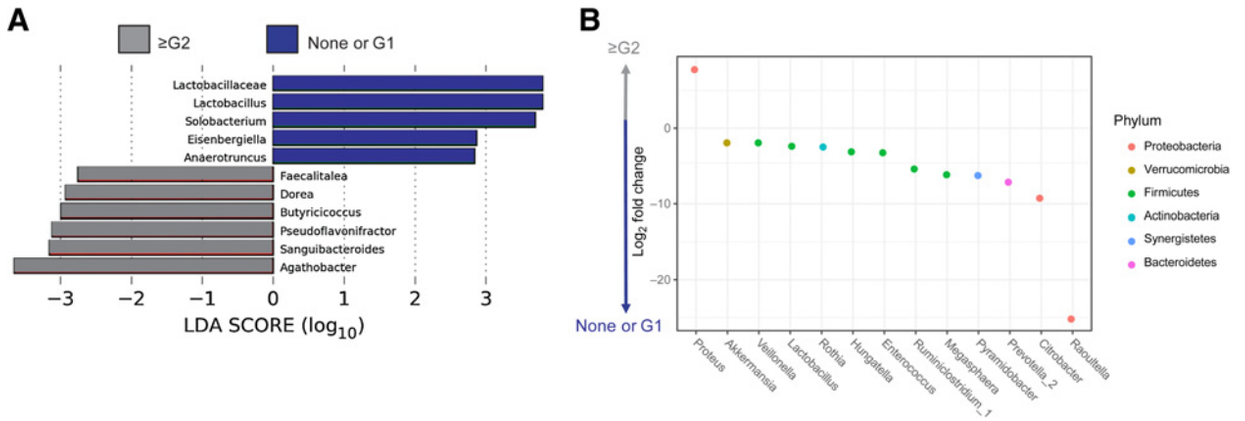

Figure 4.

GM composition and association with irAEs in ICI-treated patients with NSCLC. **A,** Differential abundance analysis using LEfSe at the genus level in antibiotic-free patients stratified by irAE severity. **B,** DESeq2 analysis at the genus-level in all patients according to irAE severity. G1, grade 1; G2, grade 2. Note that only genera that are statistically significant are reported on DESeq2 analysis.

commensals, there were higher frequencies of effector $CD4^+$ and $CD8^+$ T cells in the circulation and $CD8^+$ T-cell infiltration in the tumor. Similarly, *Ruminococcus* associates with ICI benefit in melanoma, NSCLC, and renal cell carcinoma (16, 39). For example, a mixture of 11 bacteria including *Ruminococcaceae* bacterium cv2 in preclinical models associated with high colon IFNγ production from $CD8^+$ T cells and correlated with improved anti–PD-1 efficacy (39). The positive prognostic significance of *Ruminococcaceae* was also seen in patients with melanoma who underwent neoadjuvant immune checkpoint blockade (40). Our Japanese cohort of patients with advanced NSCLC validated this association between *Ruminococcaceae* and clinical responses to anti–PD-1/PD-L1. We illustrated the potentially suppressive role of specific bacteria, such as *Lactobacillaceae, Raoultella*, and *Agathobacter* in the development of irAE. Preclinical data investigating the anti-inflammatory properties of some *Lactobacillaceae* species (41) may explain its potential modulation of off-target immune responses.

Our study has several limitations. First, we did not correlate the GM composition with a dietary history. The OS in the antibiotic-free group was numerically longer compared with those who received antibiotic (16.1 vs. 12.1 months), with no statistical significance, and this may be due to a low sample size. 16S rRNA gene sequencing may be underpowered to illustrate the whole GM signature. The use of deeper GM profiling techniques such as metagenomics sequencing is now available, and despite the current financial burden of this tool, this will likely become more affordable in the future due to increasing demand. To relay the results of GM studies to the bedside, the accessibility of this technique, including its cost and efficiency, is required.

In sum, we demonstrated the negative influence of antibiotics on GM composition and identified a differential bacteria repertoire in patients experiencing favorable clinical outcomes or low-grade irAE. Our data reinforced the importance of developing diagnostic tools aimed at identifying gut dysbiosis to predict resistance or irAE in patients with advanced NSCLC treated with ICI. Many questions remain unanswered, including whether GM composition could influence cancer incidence and severity, neoplasia histology, tumor genetics, and tumor microenvironment, and whether lines of therapy, comorbidities, comedications, and geo-distribution alter GM. Further efforts to prospectively sequence the fecal samples of patients with NSCLC are ongoing to identify specific and minimalist GM signatures associated with favorable or unfavorable clinical outcomes for ICI therapy.

Disclosure of Potential Conflicts of Interest

A. Elkrief reports grants from AstraZeneca outside the submitted work. Y. Hosomi reports personal fees from AstraZeneca, Eli Lilly Japan, Taiho Pharmaceutical, Chugai Pharmaceutical, Ono Pharmaceutical, Bristol-Myers Squibb, Kyowa Kirin, and CSL Behring outside the submitted work. L. Derosa reports other from Philantropia (PhD fellowship) and grants from Fondation Dassault outside the submitted work. L. Zitvogel is the main founder of EverImmune and reports grants and personal fees from Transgene (board of directors), and grants from Kaleido and 9 meters during the conduct of the study; personal fees from Lytix Biopharma (scientific advisory board) outside the submitted work; and patents for EP 18306282.7 licensed to EverImmune and EP 19306246.0 pending. B. Routy reports personal fees from Vedanta Company (advisory board) outside the submitted work. Y. Okuma reports grants from Grant-in-Aid for scientific research (KAKENHI) during the conduct of the study, as well as personal fees from AstraZeneca K.K, Boehringer Ingelheim Japan, Chugai Pharmaceutical Co., Ltd., Ono Pharmaceutical Co., Ltd., and Bristol-Myers Squibb outside the submitted work. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

T. Hakozaki: Resources, data curation, investigation, writing–original draft, writing–review and editing. **C. Richard:** Data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–review and editing. **A. Elkrief:** Data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–review and editing. **Y. Hosomi:** Resources, supervision, writing–review and editing. **M. Benlaïfaoui:** Data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–review and editing. **I. Mimpen:** Data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–review and editing. **S. Terrisse:** Data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–review and editing. **L. Derosa:** Data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. **L. Zitvogel:** Investigation, methodology, project administration, writing–review and editing. **B. Routy:** Data curation, software, formal analysis, supervision, validation, investigation, visualization, methodology, project administration, writing–review and editing. **Y. Okuma:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing.

Acknowledgments

This work was supported by JSPS KAKENHI (grant no.: JP19K16820). B. Routy was supported by H2020 ONCOBIOME. L. Zitvogel was supported by the Ligue contre le Cancer (équipe labelisée); Agence Nationale de la Recherche (ANR) francogermanique ANR-19-CE15-0029 and Association pour la recherche sur le cancer; Cancéropôle Ile-de-France; Fondation pour la Recherche Médicale; a donation by Elior; Fondation Carrefour; Institut National du Cancer; Inserm (HTE); the LabEx Immuno-Oncology; the RHU Torino Lumière (ANR-16-RHUS-0008); H2020 ONCOBIOME and the Seerave Foundation; the SIRIC Stratified Oncology Cell DNA Repair and Tumor Immune Elimination (SOCRATE); FHU CARE, Dassault, and Badinter philantropia; and the Paris Alliance of Cancer Research Institutes. L. Derosa's salary was supported by the RHU Torino Lumière (ANR-16-RHUS-0008) and H2020 ONCOBIOME. The authors thank TechnoSuruga Laboratory Co., Ltd. (https://www.tecsrg.co.jp/) for sequencing of DNA from fecal samples. The authors also thank Dr. Kageaki Watanabe, Dr. Kana Hashimoto, Dr. Shoko Kawai, Dr. Makiko Yomota, and other residents for patients' care and sample collection. In addition, B. Routy would like to thank the Prefontaine Family for their generous support of this project.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 11, 2020; revised May 20, 2020; accepted July 24, 2020; published first July 27, 2020.

References

1. Horn L, Spigel DR, Vokes EE, Holgado E, Ready N, Steins M, et al. Nivolumab versus docetaxel in previously treated patients with advanced non–small-cell lung cancer: two-year outcomes from two randomized, open-label, phase III trials (Checkmate 017 and Checkmate 057). J Clin Oncol 2017;35:3924–33.
2. Brahmer J, Reckamp KL, Baas P, Crinò L, Eberhardt WEE, Poddubskaya E, et al. Nivolumab versus docetaxel in advanced squamous-cell non–small-cell lung cancer. N Engl J Med 2015;373:123–35.
3. Borghaei H, Paz-Ares L, Horn L, Spigel DR, Steins M, Ready NE, et al. Nivolumab versus docetaxel in advanced nonsquamous non–small-cell lung cancer. N Engl J Med 2015;373:1627–39.
4. Rittmeyer A, Barlesi F, Waterkamp D, Park K, Ciardiello F, von Pawel J, et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. Lancet 2017;389:255–65.
5. Gandhi L, Rodriguez-Abreu D, Gadgeel S, Esteban E, Felip E, De Angelis F, et al. Pembrolizumab plus chemotherapy in metastatic non-small-cell lung cancer. N Engl J Med 2018;378:2078–92.
6. Reck M, Rodriguez-Abreu D, Robinson AG, Hui R, Csoszi T, Fulop A, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. N Engl J Med 2016;375:1823–33.
7. Herbst RS, Baas P, Kim DW, Felip E, Perez-Gracia JL, Han JY, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. Lancet 2016;387:1540–50.
8. Doroshow DB, Sanmamed MF, Hastings K, Politi K, Rimm DL, Chen L, et al. Immunotherapy in non–small cell lung cancer: facts and hopes. Clin Cancer Res 2019;25:4592–602.
9. Socinski MA, Jotte RM, Cappuzzo F, Orlandi F, Stroyakovskiy D, Nogami N, et al. Atezolizumab for first-line treatment of metastatic nonsquamous NSCLC. N Engl J Med 2018;378:2288–301.
10. Rizvi NA, Hellmann MD, Snyder A, Kvistborg P, Makarov V, Havel JJ, et al. Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. Science 2015;348:124–8.
11. Cristescu R, Mogg R, Ayers M, Albright A, Murphy E, Yearley J, et al. Pan-tumor genomic biomarkers for PD-1 checkpoint blockade–based immunotherapy. Science 2018;362:eaar3593.
12. Komiya K, Nakamura T, Abe T, Ogusu S, Nakashima C, Takahashi K, et al. Discontinuation due to immune-related adverse events is a possible predictive factor for immune checkpoint inhibitors in patients with non-small cell lung cancer. Thorac Cancer 2019;10:1798–804.
13. Vetizou M, Pitt JM, Daillere R, Lepage P, Waldschmitt N, Flament C, et al. Anticancer immunotherapy by CTLA-4 blockade relies on the gut microbiota. Science 2015;350:1079–84.
14. Matson V, Fessler J, Bao R, Chongsuwat T, Zha Y, Alegre ML, et al. The commensal microbiome is associated with anti–PD-1 efficacy in metastatic melanoma patients. Science 2018;359:104–8.
15. Chaput N, Lepage P, Coutzac C, Soularue E, Le Roux K, Monot C, et al. Baseline gut microbiota predicts clinical response and colitis in metastatic melanoma patients treated with ipilimumab. Ann Oncol 2017;28:1368–79.
16. Routy B, Le Chatelier E, Derosa L, Duong CPM, Alou MT, Daillere R, et al. Gut microbiome influences efficacy of PD-1-based immunotherapy against epithelial tumors. Science 2018;359:91–7.
17. Gopalakrishnan V, Spencer CN, Nezi L, Reuben A, Andrews MC, Karpinets TV, et al. Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients. Science 2018;359:97–103.
18. Zheng Y, Wang T, Tu X, Huang Y, Zhang H, Tan D, et al. Gut microbiome affects the response to anti-PD-1 immunotherapy in patients with hepatocellular carcinoma. J Immunother Cancer 2019;7:193.
19. Elkrief A, Derosa L, Kroemer G, Zitvogel L, Routy B. The negative impact of antibiotics on outcomes in cancer patients treated with immunotherapy: a new independent prognostic factor? Ann Oncol 2019;30:1572–9.
20. Wilson BE, Routy B, Nagrial A, Chin VT. The effect of antibiotics on clinical outcomes in immune-checkpoint blockade: a systematic review and meta-analysis of observational studies. Cancer Immunol Immunother 2020;69:343–54.
21. Pinato DJ, Howlett S, Ottaviani D, Urus H, Patel A, Mineo T, et al. Association of prior antibiotic treatment with survival and response to immune checkpoint inhibitor therapy in patients with cancer. JAMA Oncol 2019;5:1774–8.
22. Wang Y, Wiesnoski DH, Helmink BA, Gopalakrishnan V, Choi K, DuPont HL, et al. Fecal microbiota transplantation for refractory immune checkpoint inhibitor-associated colitis. Nat Med 2018;24:1804–8.
23. Takahashi S, Tomita J, Nishioka K, Hisada T, Nishijima M. Development of a prokaryotic universal primer for simultaneous analysis of Bacteria and Archaea using next-generation sequencing. PLoS One 2014;9:e105592.
24. Wang Q, Garrity GM, Tiedje JM, Cole JR. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 2007;73:5261–7.
25. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: high-resolution sample inference from Illumina amplicon data. Nat Methods 2016;13:581–3.
26. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 2013;41:D590–6.
27. McMurdie PJ, Holmes S. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. PLoS One 2013;8:e61217.
28. Chao A, Chazdon RL, Colwell RK, Shen TJ. A new statistical approach for assessing similarity of species composition with incidence and abundance data. Ecology letters 2005;8:148–59.
29. Lozupone C. UniFrac KR: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol 2005;71:8228–35.
30. Zhu C, Yu J. Nonmetric multidimensional scaling corrects for population structure in association mapping with different sample types. Genetics 2009;182:875–88.
31. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol 2011;12:R60.
32. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 2014;15:550.
33. Eisenhauer EA, Therasse P, Bogaerts J, Schwartz LH, Sargent D, Ford R, et al. New response evaluation criteria in solid tumours: revised RECIST guideline (version 1.1). Eur J Cancer 2009;45:228–47.
34. National Institutes of Health. Common Terminology Criteria for Adverse Events (CTCAE) v4.0. Bethesda (MD): National Institutes of Health, National Cancer Institute; 2010. Available from: http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm#ctc_40.
35. Kaplan EL, Meier P. Nonparametric estimation from incomplete observations. J Am Statist Assoc 1958;53:457–81.

36. Mantel N. Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 1966; 50:163–70.
37. Jin Y, Dong H, Xia L, Yang Y, Zhu Y, Shen Y, et al. The diversity of gut microbiome is associated with favorable responses to anti-programmed death 1 immunotherapy in Chinese patients with NSCLC. J Thorac Oncol 2019;14:1378–89.
38. Derosa L, Routy B, Fidelle M, Iebba V, Alla L, Pasolli E, et al. Gut bacteria composition drives primary resistance to cancer immunotherapy in renal cell carcinoma patients. Eur Urol 2020;78:195–206.
39. Tanoue T, Morita S, Plichta DR, Skelly AN, Suda W, Sugiura Y, et al. A defined commensal consortium elicits CD8 T cells and anti-cancer immunity. Nature 2019;565:600–5.
40. Batten M, Shanahan E, Simpson R, Read M, Silva IP, Angelatos A, et al. Gut microbiota predicts response and toxicity with neoadjuvant immunotherapy. In: Proceedings of the 2020 AACR Annual Virtual Meeting I; 2020 Apr 27–28; session VMS.TB06.02 5734. Philadelphia (PA): AACR; 2020.
41. Kim JA, Kim SH, Kim IS, Yu DY, Kim SC, Lee SH, et al. Anti-inflammatory effects of a mixture of lactic acid bacteria and sodium butyrate in atopic dermatitis murine model. J Med Food 2018;21:716–25.