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Intestinal flora characteristics of advanced non-small cell lung cancer in China and their role in chemotherapy based on metagenomics: A prospective exploratory cohort study

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Abstract

Background: Lung cancer has the highest mortality rate among malignant tumors, with non-small cell lung cancer (NSCLC) being the most common type. As the main component of the human microflora, the intestinal flora interacts with the human body to affect immunity, metabolism, and the formation of diseases.

Methods: Forty-five patients with advanced NSCLC who received platinum-containing dual-drug chemotherapy were enrolled in a prospective exploratory cohort study. The intestinal flora was dynamically collected at baseline and after two chemotherapy cycles. Next-generation sequencing and metagenomics were then used to analyze the species and function of the intestinal flora at all levels.

Results: Significant differences in the intestinal flora of patients with NSCLC were found according to sex and age. At the family level, the abundances of *Streptococcaceae*, *Lactobacillaceae*, and *Leuconostocaceae* after platinum-containing dual-drug chemotherapy were significantly higher compared to those before chemotherapy. At the family level, patients with chemotherapy-induced gastrointestinal reactions had a significantly higher abundance of *Leuconostocaceae* than those without gastrointestinal responses. Meanwhile, patients with gastrointestinal reactions had higher metabolism, human diseases, cellular processes, and environmental information processing than those who did not. At the genus level, responders had higher abundances of *Bacteroides* compared to nonresponders. Moreover, nonresponders had higher levels of the six major metabolic pathways compared to responders.

Conclusions: The intestinal flora of Chinese patients with advanced NSCLC differed according to sex and age. Moreover, significant differences in the intestinal flora were noted after chemotherapy, which could be associated with chemotherapy-induced gastrointestinal reactions and the efficacy of chemotherapy.

KEYWORDS
chemotherapy, intestinal flora, metagenomics, NSCLC

INTRODUCTION

Lung cancer is one of the most common malignant tumors. According to the GLOBOCAN estimates issued by the International Agency for Research on Cancer of the World

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Health Organization, lung cancer had a global incidence and mortality rate of 11.4% and 18.0% in 2020, respectively. It has become the leading cause of death from malignant tumors,[1] with non-small cell lung cancer (NSCLC) being one of the most common types, accounting for approximately 85% of lung cancer cases.[2] Lung cancers are usually hidden, with most patients already presenting with advanced stage disease upon diagnosis. Although targeted therapies and immunotherapy have considerably improved the quality of life and survival of patients with NSCLC, 60% of patients with lung cancer have no driver gene mutations that can accept targeted therapy. By contrast, immunotherapy response rates among patients with NSCLC only range from 15% to 25%.[3–5] Thus, chemotherapy remains the cornerstone of the treatment for advanced NSCLC. Since the advent of targeted therapy and immunotherapy, research and innovation in the field of conventional cytotoxic chemotherapy drugs have substantially slowed down.

Human intestinal flora, the main component of human microflora, is a complex system that facilitates the formation of a stable nutritional balanced microenvironment and interacts with the human body to affect immunity, metabolism, and disease formation.[6–8] The intestinal flora has been associated with gastrointestinal diseases (e.g., inflammatory bowel disease) and nongastrointestinal diseases, such as obesity, cardiovascular disease, and diabetes.[9–11] Currently, increasing evidence has emphasized the role of intestinal microbes in tumor treatment. It has been previously reported that intestinal microbe regulation may affect the response to several tumor treatments.[12] The bacterial population that resides specifically within the tumor exhibits tumor-type specificity. Moreover, mechanism studies have shown that microbiota plays an essential role in the occurrence, development, treatment, and prognosis of tumors.[13,14]

At present, most research hotspots related to intestinal flora and tumors focus on immunotherapy, with more in-depth research into melanoma having emerged. Some researchers compared the growth of melanoma in mice with different symbiotic flora and confirmed that bifidobacteria exerted antitumor effects through 16S ribosomal RNA sequencing. In fact, oral administration of bifidobacteria was found to enhance dendritic cell function, facilitating the initiation and aggregation of CD8+ T cells in the tumor microenvironment to the same extent as PD-L1-specific antibody therapy. Evidence suggests that *Bifidobacterium*-combined immunotherapy can almost eliminate tumor growth and that cancer immunotherapy can be improved by adjusting the microbiota.[15] Moreover, Routy et al. found that the abundance of Akkermansia mucous was associated with the effects of immunotherapy in patients undergoing fecal genomics analysis before treatment.[16] Several researchers who analyzed the intestinal flora of melanoma patients treated with PD-1 inhibitors showed that responders had a greater diversity of intestinal flora and that the α-diversity of stool samples was positively correlated with progression-free survival rate. Another study found that the content of Fischer bacteria was significantly positively correlated with the progression-free survival period.[17] As such, this study explored the intestinal flora characteristics of patients with advanced NSCLC and its role in platinum-containing dual-drug chemotherapy based on metagenomics.

METHODS

Patients

Forty-five patients diagnosed with NSCLC through cytology or histology between July 2018 and November 2019 were enrolled in a prospective exploratory cohort study. All patients received single- or dual-drug combination chemotherapy for the first time. None of the patients had second primary malignancy and digestive system disease. Furthermore, patients who had taken antibiotics, laxatives, immunosuppressants, and probiotics for a long time were excluded. This study was approved by the institutional ethics committee of The Second Hospital of Dalian Medical University (no. 52, 2020) and was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Sample collection

Fecal samples were collected from the study subjects after patients provided their informed consent following the principle of voluntariness. In the lung cancer group, fresh fecal samples were collected from all 45 patients (A) before chemotherapy and from 21 patients (B) after chemotherapy (Figure 1). After collection, the samples were frozen and stored in a refrigerator at −80°C.

Extraction and purification of fecal DNA

Fecal DNA was extracted using the cetyltrimethylammonium bromide method. For DNA purification, the degree of

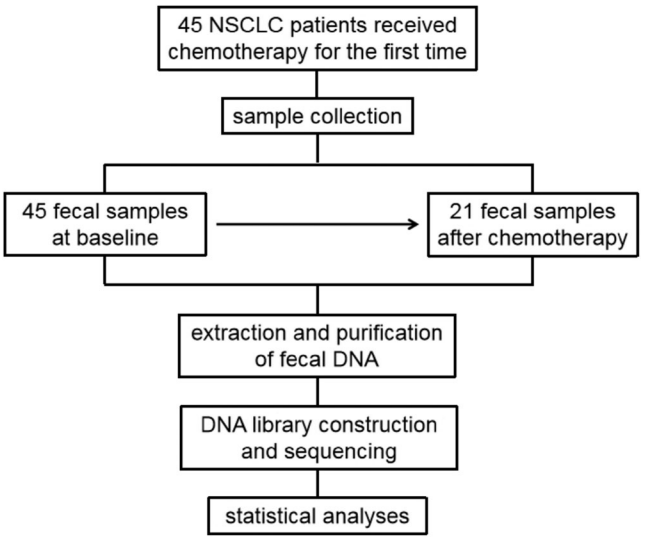

FIGURE 1 Study scheme

DNA degradation and potential contamination was initially assessed on 1% agarose gel. After that, the DNA concentration was measured using a dsDNA analysis kit with a Qubit 2.0 Fluorometer (California Life Technologies). Only DNA samples with an optical density value of 1.8–2.0 and a DNA content above 1 μg were used to construct the library.

DNA library construction and sequencing

After constructing the library, each sample with a total DNA amount of 1 μg was used as an input material for DNA sample preparation. NEBNext Ultra was used as the input mate (Illumina) to generate a sequencing library and add an index code to the attribute sequence of each sample. Briefly, DNA sample fragments were sonicated and connected to a full-length adapter for Illumina sequencing before further amplification by polymerase chain reaction (PCR). Finally, PCR products were purified using the AMPure XP system. The size distribution of the library was analyzed using the Agilent 2100 Bioanalyzer and quantified using real-time PCR. After the DNA library passed the test, sequencing was performed on the Illumina HiSeq platform, and paired-end reads were generated. These activities were detected according to the previous method.[18]

Statistical analysis

Raw data obtained by the Illumina HiSeq sequencing platform were preprocessed using Readfq. When contamination was found, raw data were compared with the host database to obtain valid data. The SOAPdenovo software (BGI, China) was used for the assembly of preprocessed data for metagenomic analysis. MetaGeneMark was used to predict the open reading frame of each sample, and redundant data were removed using CD-HIT software (Weizhong Li, Philadelphia, USA).[19] The abundance information for each sample was calculated. The functional genes were compared with the microbial population extracted from the nonredundant (NR) protein sequence database. The filtered species was analyzed using the least common ancestor algorithm to determine the species annotation information of the sequence. On the basis of the results and gene abundance table, each sample's abundance information and gene number table at each classification level were obtained. For abundance tables, Krona analysis, principal component analysis, and nonmetric multidimensional scaling dimensionality reduction analyses were performed. Analysis of similarities was used to test for differences between groups, after which differential species were identified using Metastats and linear discriminant analysis effect size (LEfSe). These activities were detected according to a previous method by Zhang et al.[18]

RESULTS

Basic clinical information of patients with NSCLC

Our study subjects consisted of 45 patients (average age, 63.4 years; including 15 female patients and 30 male patients) with stage IIIB–IV NSCLC who received platinum-containing dual-drug chemotherapy (Table 1). All patients had ECOG scores of 0–1 points. Among the included patients, 11 had squamous cell carcinoma and received a chemotherapy regimen comprising gemcitabine combined with platinum (cisplatin or carboplatin), whereas 34 had adenocarcinoma and received a chemotherapy regimen containing pemetrexed combined with platinum (cisplatin or carboplatin). Chemotherapy-induced gastrointestinal reactions were observed in 42% of the included patients.

TABLE 1 Baseline demographic and clinical characteristics of the study subjects

Patient characteristics	Value
N	**45**
Age	
Mean (SD)	63.4
Range	47–77
Gender (%)	
Female	15 (33%)
Male	30 (67%)
Smoking history (%)	
Nonsmoker	22 (44%)
Smoker	23 (46%)
Stage at entry (%)	
IIIB–IIIC	8 (18%)
IV	37 (82%)
Histology (%)	
Squamous carcinoma	11 (24%)
Adenocarcinoma	34 (76%)
ECOG (%)	
0	17 (38%)
1	28 (62%)
Gastrointestinal reactions (%)	
Yes	19 (42%)
No	26 (58%)
RECIST	
CR	0
PR	7
SD	23
PD	10
No evaluation	5

Differences in intestinal flora according to sex

The included patients were grouped according to sex, with A1 and A2 indicating females and males. We initially started from the relative abundance table at the family level and selected the top 10 species with the largest relative abundance in each sample. Figure 2(a) shows that regardless of sex, the top 10 species had similar relative abundance. Among these 10 species, *Bacteroidaceae*, *Rikenellaceae*, *Ruminococcaceae*, and *Selenomonadaceae* were relatively abundant in female patients' intestinal flora, whereas *Prevotellaceae*, *Lachnospiraceae*, *Enterobacteriaceae*, *Streptococcaceae*, *Eubacteriaceae*, and *Bifidobacteriaceae* were relatively abundant in male patient intestinal flora (Figures 2(a, b)).

Differences in intestinal flora according to age

Patients were divided into group A (under 60 years old) and group B (over 60 years old) to explore whether the intestinal flora differed according to age. Initially, grouping analysis was conducted, with our results suggesting that our grouping was meaningful at the species level ($R > 0$, $p < 0.05$) (Figure 3(a)). At the species level, the top 10 species with the largest relative abundance were similar across different ages (Figure 3(b)). Patients under 60 years of age had a higher relative abundance of *Prevotella copri*, *Faecaliacterium prausnitzii*, *Firmicutes bacterium* CAG:83, *Clostridium hiranonis*, *Fusobacterium mortiferum*, and *Ruminococcus* sp. CAG:177 compared to those over 60. Conversely, patients over 60 years old had a higher relative abundance of *Bacteroides stercoris*, *Eubacterium eligens*, and *Bacteroides plebeius* than those under 60 (Figure 3(c)). Moreover, our results found that at the species level, patients over 60 years old had significantly greater *Bacteroides* sp. 31 40A (Figure 3(d)) and *Bacteroides vulgatus* (Figure 3(e)) compared to those under 60 years old, with this difference being statistically significant through the Metastats analysis of the different species between the groups. It can be seen that age may also be one of the influencing factors of intestinal flora in NSCLC patients.

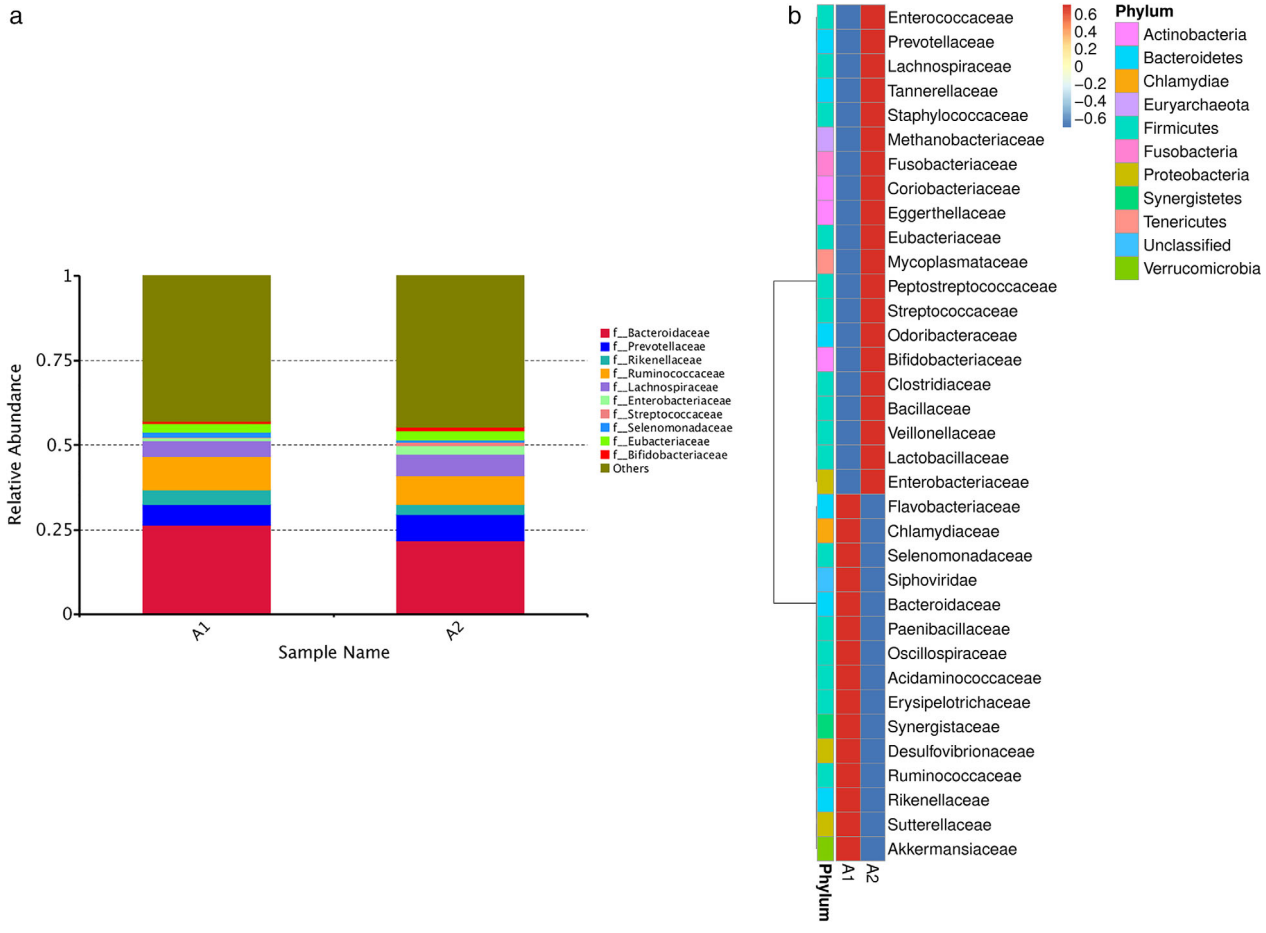

FIGURE 2 (a) At the family level, the top 10 species with the largest relative abundance in all samples are shown, with "Others" representing other species detected. In the figure, the vertical axis represents the relative proportion of the species annotated to a particular type, whereas the horizontal axis represents the sample name. The species category corresponding to each color block is shown in the legend on the right. (b) Cluster diagrams showing species abundance at the family level. The horizontal axis shows the grouping information, whereas the vertical axis shows the annotation information, with a species-clustering tree presented on the left

Differences in intestinal flora before and after chemotherapy

Stool samples from patients with NSCLC were grouped according to the timing of sampling: before treatment (group A1) and after treatment (group A2). Initially, we calculated the number of genes in each sample species at the family level. Accordingly, *Bacteroidaceae*, *Lachnospiraceae*, *Clostridiaceae*, and *Ruminococcaceae* had the highest number of genes before and after chemotherapy (Figure 4(a)). After

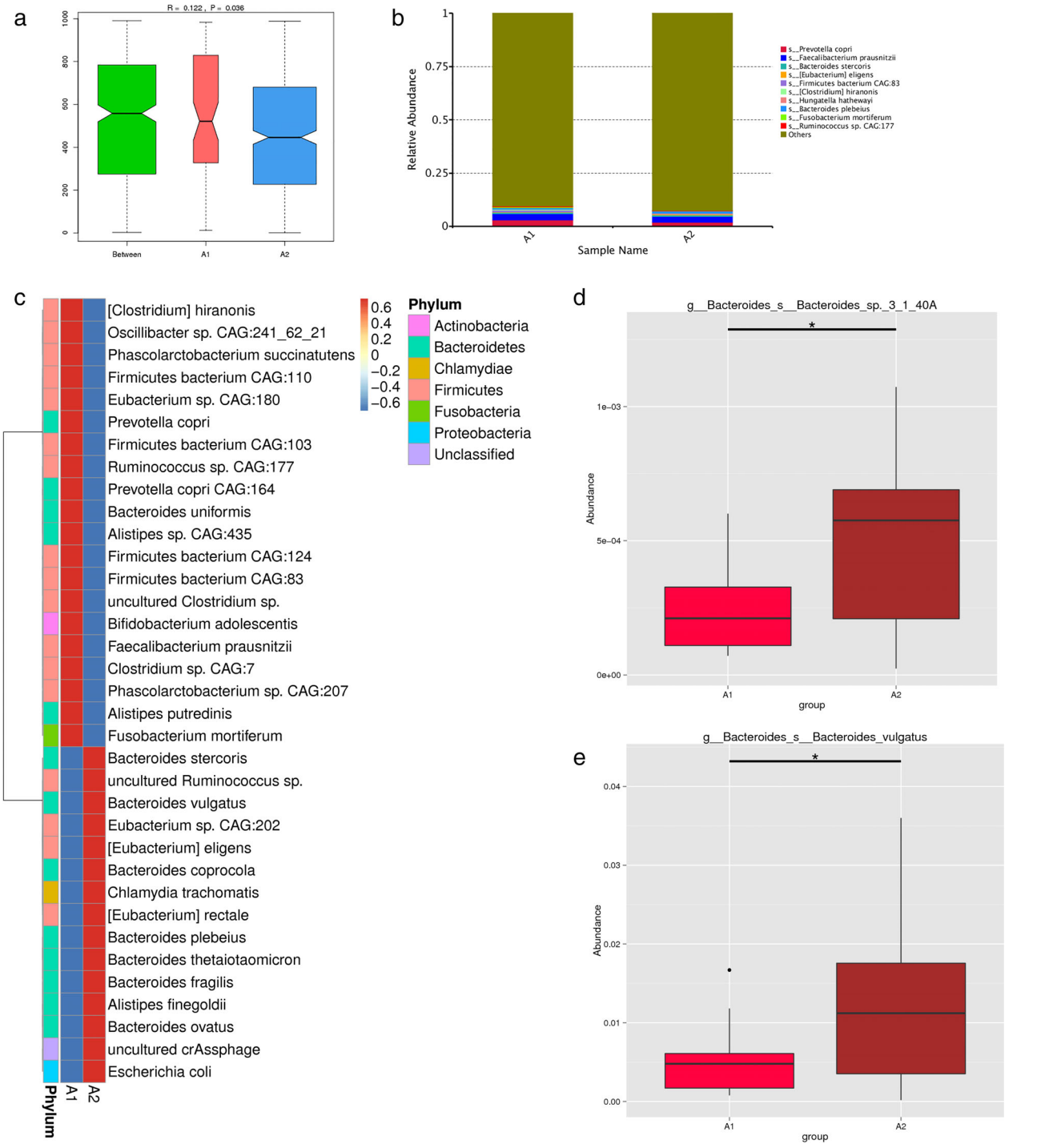

FIGURE 3 (a) Similarity analysis box plot based on species level. The horizontal axis indicates the grouping information, whereas the vertical axis indicates the distance information, with two sets of merged details in between. (b) At the species level, the top 10 species with the largest relative abundance in all samples are shown, with "Others" representing other species detected. In the figure, the vertical axis represents the relative proportion of the species annotated to a particular type, whereas the horizontal axis represents the sample name. The species category corresponding to each color block is shown in the legend on the right. (c) Cluster diagrams showing species abundance at the species level. The horizontal axis shows the grouping information, whereas the vertical axis shows the annotation information, with a species-clustering tree presented on the left. (d) and (e) Box plots showing significant differences in abundance at the species level. The horizontal axis indicates the sample grouping, whereas the vertical axis indicates the relative abundance of the corresponding species. *Indicates a significant difference between the two groups ($p < 0.05$)

that, starting from the relative abundance of species at the family level, the top 10 species with the largest relative abundance in each sample were selected. Our findings subsequently showed that the top 10 species with the largest relative abundance were the same before and after chemotherapy (Figure 4(b)). Figure 4(c) shows a cluster diagram of the difference in species abundance of the intestinal flora before and after chemotherapy at the departmental level. Metastatic analysis of the different species between the groups found that the abundances of *Streptococcaceae* (Figure 4(d)), *Lactobacillaceae* (Figure 4(e)), and *Leuconostocaceae* (Figure 4(f)) were significantly greater after chemotherapy than those before chemotherapy ($p < 0.05$).

Correlation between chemotherapy-induced gastrointestinal reactions and intestinal flora

According to whether gastrointestinal reactions occurred during chemotherapy, samples were divided into those with (group A1, 19 samples) and without gastrointestinal reactions (group A2, 26 samples). In patients with and without gastrointestinal reactions, common intestinal flora genes accounted for the majority at baseline, with only minute differences (Figure 5(a)). Subsequently, we analyzed the species abundance of each sample at the family level (Figure 5(b)) and found that patients with gastrointestinal reactions had a significantly higher abundance of *Leuconostocaceae* compared to those without gastrointestinal reactions ($p < 0.05$; Figure 5(c)). Moreover, the KEGG database was used to analyze the relationship between chemotherapy-induced gastrointestinal reactions and intestinal flora from the biological metabolic pathway. Among the six major metabolic pathways, metabolism, human diseases, cellular processes, and environmental information processing pathways were more abundant in patients with gastrointestinal reactions compared to those without the same (Figure 5(d, e)).

Correlation between the intestinal flora and efficacy of chemotherapy

Chemotherapy efficacy was evaluated after two cycles. Patients with NSCLC whose efficacy was assessed as SD, PR, and CR were classified as responders (group A1), whereas those whose efficacy was assessed as PD were classified as nonresponders (group A2). We started from the relative abundance of species at the family level and selected the top 10 species with the highest relative abundance in each sample. Accordingly, both groups were similar in terms of the top 10 species with the highest relative abundance (Figure 6(a)). Figure 6(b) presents a cluster diagram of the difference in species abundance at the species level. Metastatic analysis of the different species between the groups at the species level revealed that responders had higher abundances of *Bacteroides coprocola* (Figure 6(c)), *Bacteroides intestinalis* (Figure 6(d)), *Bacteroides fluxus* (Figure 6(e)), and uncultured *Bacteroides* sp. (Figure 5(f)) compared to nonresponders.

These intestinal florae belong to the genus *Bacteroides*. Analysis of the KEGG database found that nonresponders had higher abundances of the six major metabolic pathways (metabolism, human diseases, cellular processes, environmental information processing, genetic information processing, and organic systems) (Figure 6(g, h)).

DISCUSSION

Chemotherapy is often used as an adjuvant treatment for patients with lung cancer or as the primary treatment for patients with advanced lung cancer. The first-line chemotherapy for advanced NSCLC includes a combination of platinum (referring to cisplatin and carboplatin). The current study employed metagenomics to examine the intestinal flora of 45 patients with advanced NSCLC who received first-line chemotherapy. Several researchers who explored the characteristics of the intestinal flora of patients with lung cancer found that the specific intestinal microbiome of such patients might be associated with chemotherapy outcomes.[20] After analyzing the intestinal flora of patients with advanced NSCLC before and after chemotherapy, the current study found that the top 10 species with the largest relative abundance remained the same after chemotherapy. However, the abundances of *Streptococcaceae*, *Lactobacillaceae*, and *Leuconostocaceae* were significantly higher after than before chemotherapy.

Patients with lung cancer often exhibit intestinal severe adverse reactions, such as chemotherapy-related diarrhea and constipation, during the course of platinum-containing dual-drug chemotherapy. One study in patients with SCLC who received irinotecan combined with cisplatin showed that those with chemotherapy-related diarrhea developed intestinal flora disorders and decreased intestinal flora diversity.[21] Moreover, some researchers found that intestinal flora was associated with gastrointestinal reactions in patients receiving chemotherapy for lung cancer. One study showed that *Clostridium butyricum* reduces chemotherapy-related diarrhea, decreases the systemic inflammatory response, and encourages the maintenance of homeostasis in patients with lung cancer.[22] Pemetrexed further increased the relative abundance of *Enterobacteriaceae* and *Phyllanaceae* (i.e., *Enterococcus*, *Lactobacillus*, and *Streptococcus*) and significantly altered the integrity of the epithelial barrier, which was associated with early inflammation. This preliminary study showed that the correlation between lung tumor transplants and pemetrexed promoted changes in microbiota composition. This information increases our understanding of the effects of chemotherapy on microbiota, which may help in minimizing side effects and improving treatment effects in the future.[23] Our study found that patients with NSCLC who developed gastrointestinal reactions had a significantly higher abundance of *Leuconostocaceae* than those without gastrointestinal responses, suggesting that

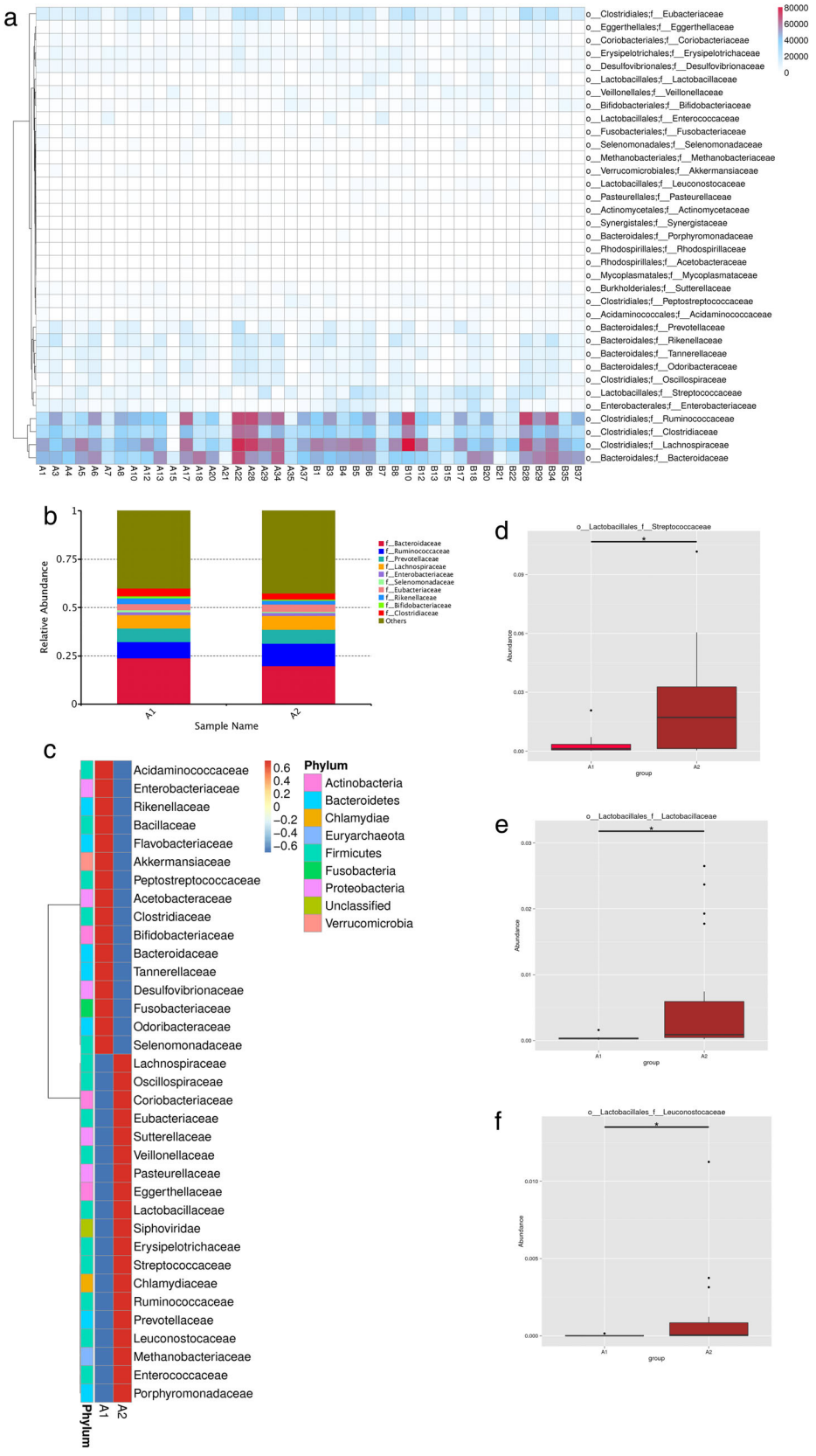

FIGURE 4 (a) Species abundance-cluster heat map based on the number of genes at the family level. The horizontal indicates the sample information in each graph, whereas the vertical indicates the species annotation information. The cluster tree on the left of the figure is the species cluster tree, whereas the middle heat map corresponds to the number of genes in each row of species in the corresponding sample. The number of genes corresponding to each color is shown in the legend on the right. (b) At the family level, the top 10 species with the largest relative abundance in all samples are shown, with "Others" representing other species detected. In the figure, the vertical axis represents the relative proportion of the species annotated to a particular type, whereas the horizontal axis represents the sample name. The species category corresponding to each color block is shown in the legend on the right. (c) Cluster diagrams showing species abundance at the family level. The horizontal axis shows the grouping information, whereas the vertical axis shows the annotation information, with a species-clustering tree presented on the left. (d–f) Box plots showing significant differences in abundance at the family level. The horizontal axis indicates the sample grouping, whereas the vertical axis indicates the relative abundance of the corresponding species. *Indicates a significant difference between the two groups ($p < 0.05$)

Leuconostocaceae may also be associated with chemotherapy-induced gastrointestinal responses, which can help in reducing the same.

Lewis lung cancer mouse models have been used in some studies to investigate the antitumor effects of the intestinal flora. Accordingly, mice treated with cisplatin

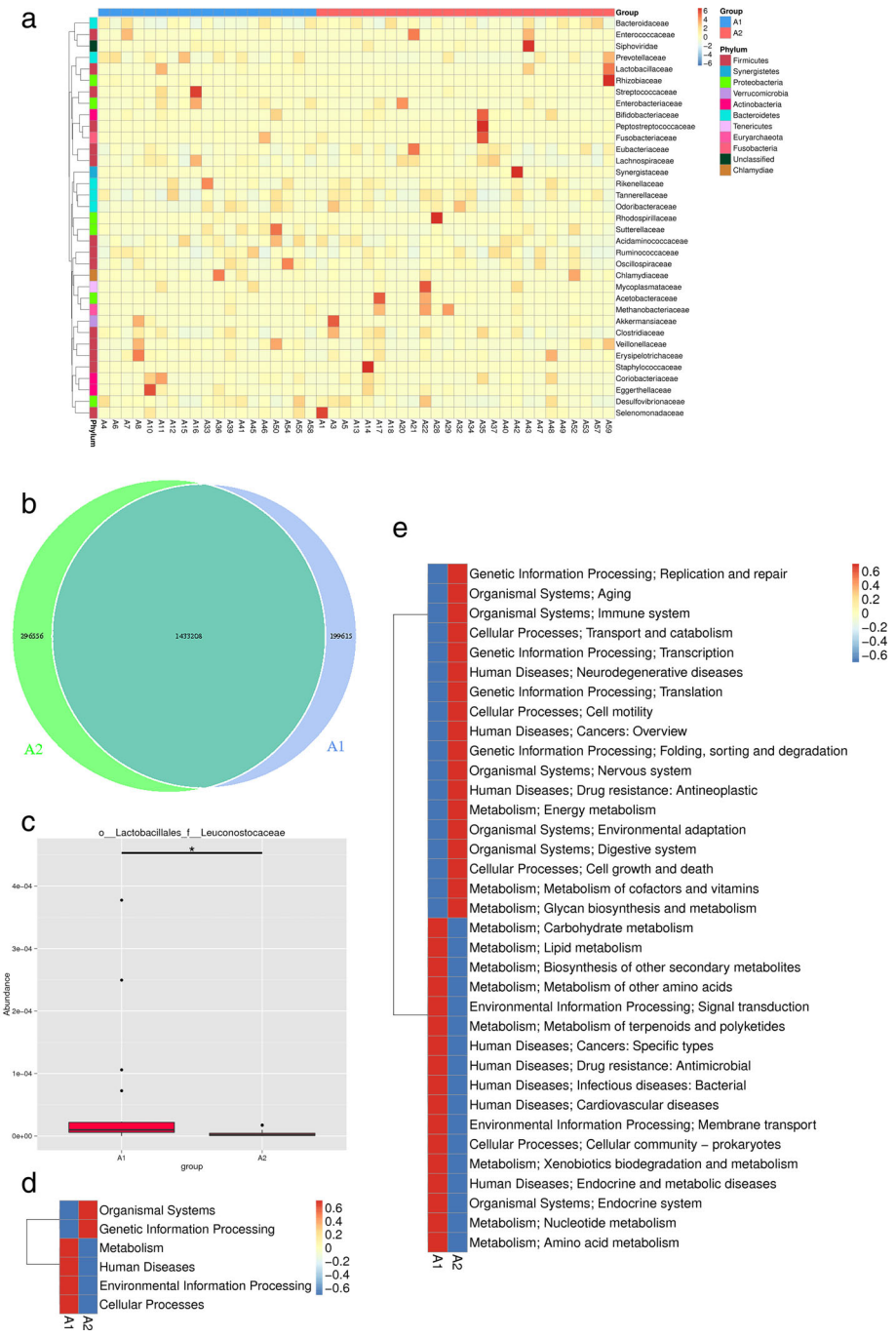

FIGURE 5 (a) Cluster diagram showing species abundance at the phylum and genus levels. In each diagram, the horizontal axis indicates the sample information, whereas the vertical axis indicates the species annotation information, with a species-clustering tree presented on the left. The value corresponding to the middle heat map is the Z-value obtained by normalizing the relative abundance of each row of species. (b) Venn diagram for distributing common genes in the intestinal flora of patients with and without gastrointestinal reactions. (c) Box plots showing significant differences in abundance at the family level. The horizontal axis indicates the sample grouping, whereas the vertical axis indicates the relative abundance of the corresponding species. *Indicates a significant difference between the two groups ($p < 0.05$). d and e: The abundance cluster heat map of the six major metabolic pathways and submetabolic pathways in the two groups

combined with ABX (an antibiotic mixture of vancomycin, ampicillin, and neomycin) exhibited destruction of their symbiotic flora and had larger tumor sizes, as well as significantly lower survival rates, compared to those treated with cisplatin alone. By contrast, mice treated with cisplatin combined with *Lactobacillus* bacteria displayed smaller tumors and higher survival rates. Further studies have shown that ABX can partially disrupt cisplatin function by upregulating VEGFA expression and downregulating BAX and CDKN1B expression. Moreover, ABX reduced the expressions of IFN-γ, GZMB, and PRF1 in CD8(+) T cells in these mice, indicating that the symbiotic flora had an immune-enhancing effect. By contrast, mice cotreated with *Lactobacillus* showed an enhanced antitumor response and upregulated IFN-γ, GZMB, and PRF1 expression.[24] Thus, evidence has shown that particular specific intestinal flora can enhance the antitumor effect of chemotherapeutics. Taken together, our study found that responders had a higher abundance of *Bacteroides coprocola*, *Bacteroides intestinalis*, *Bacteroides fluxus*, and uncultured *Bacteroides* sp. compared to nonresponders, all of which belong to the genus *Bacteroides*. Moreover, our findings showed that nonresponders showed a greater abundance of the six major metabolic pathways than responders. This suggests

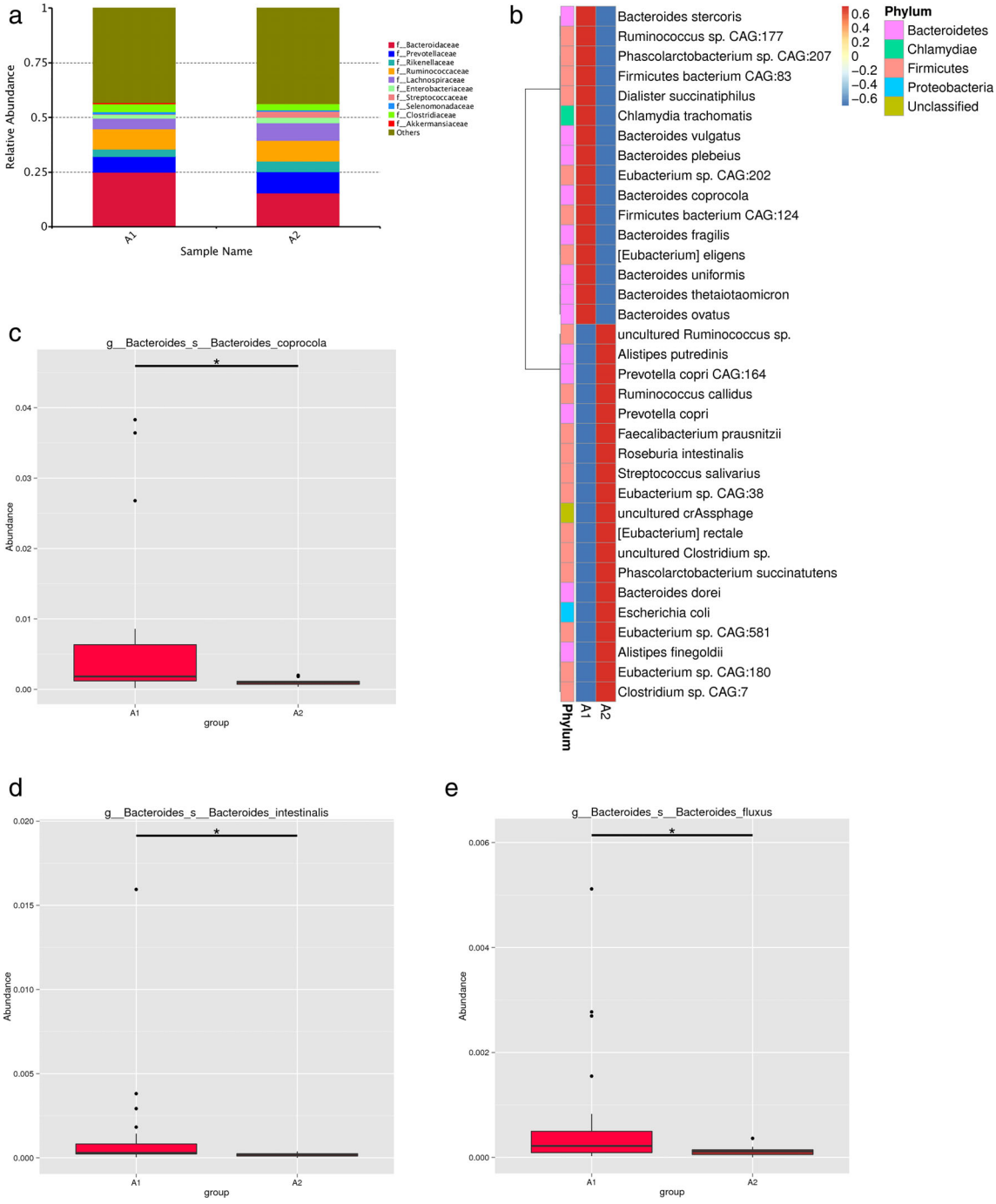

FIGURE 6 (a) At the species level, the top 10 species with the largest relative abundance in all samples are shown, with "Others" representing other species detected. In the figure, the vertical axis represents the relative proportion of the species annotated to a particular type, whereas the horizontal axis represents the sample name. The species category corresponding to each color block is shown in the legend on the right. (b) Cluster diagrams showing species abundance at the species level. The horizontal axis shows the grouping information, whereas the vertical axis shows the annotation information, with a species-clustering tree presented on the left. (c–f) Box plots show species with significant differences in abundance at the species level. The horizontal axis represents the sample grouping, whereas the vertical axis represents the relative abundance of the corresponding species. *Indicates a significant difference between the two groups ($p < 0.05$). (g and h) Abundance cluster heat map of the six major metabolic pathways and submetabolic pathways in the two groups

that patients with lung cancer with a high abundance of *Bacteroides* had a better response to platinum-containing dual-drug chemotherapy. Notably, significant differences in the intestinal flora of patients with NSCLC were noted after chemotherapy and were associated with chemotherapy-induced gastrointestinal reactions and chemotherapy efficacy.

Our data show that the intestinal flora of Chinese patients with advanced NSCLC differed according to sex and age. Moreover, significant differences in the intestinal flora were

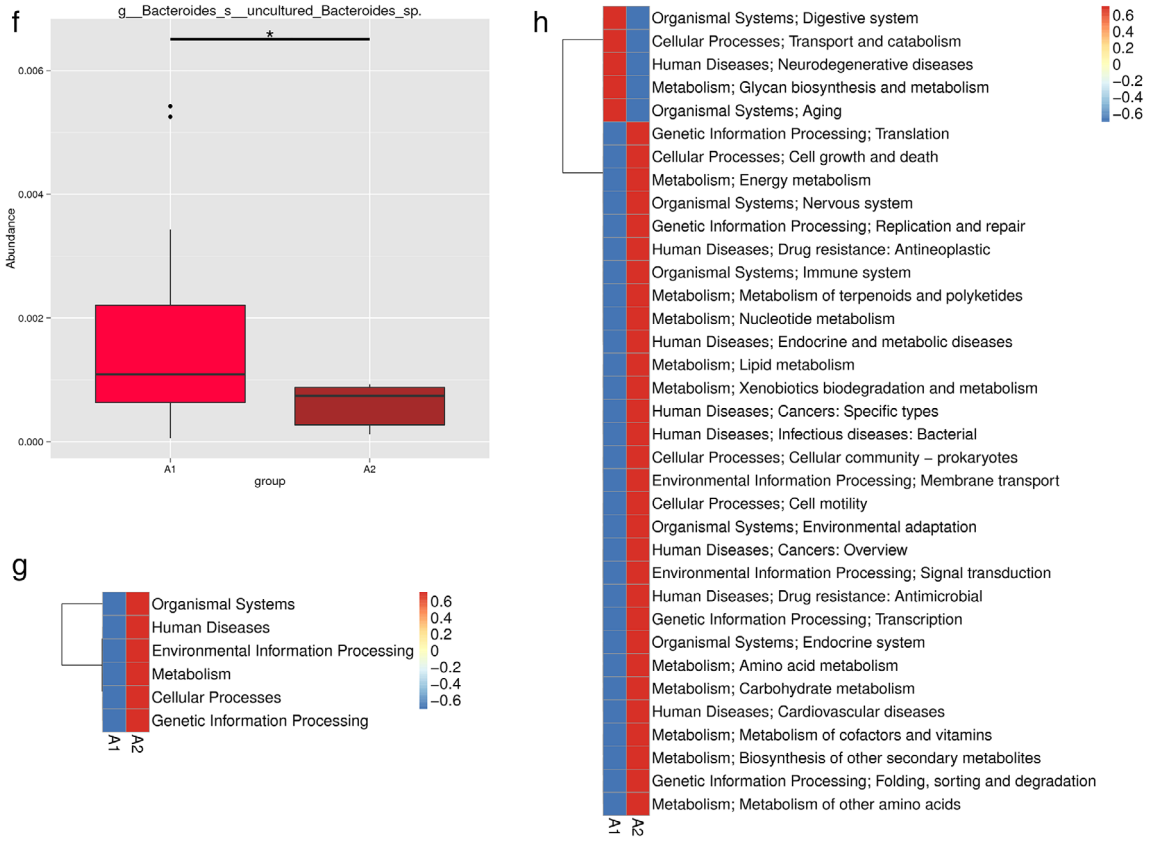

FIGURE 6 (Continued)

noted after chemotherapy, which could be associated with chemotherapy-induced gastrointestinal reactions and the efficacy of chemotherapy. However, this study still has some shortcomings and limitations. First, diet may also affect the type and abundance of some intestinal flora, which was not considered in this study. Second, the number of patients enrolled in this subject was relatively small, and only 21 samples were collected after chemotherapy. It is therefore necessary to expand the sample size to improve the accuracy of the results. In addition, the genomics and metabolomics of intestinal flora are still being researched, and we look forward to reporting further progress in the association between lung cancer and the intestinal flora in the future.

In conclusion, the intestinal flora of Chinese patients with advanced NSCLC differed according to sex and age. Moreover, significant differences in the intestinal flora were noted after chemotherapy, which could be associated with chemotherapy-induced gastrointestinal reactions and the efficacy of chemotherapy.

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CONFLICT OF INTEREST

The authors have nothing to declare.

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