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Original Research Article

Altered interaction network in the gut microbiota of current cigarette smokers

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ABSTRACT

The association between cigarette smoking and the gut microbiota remains unclear, and there is no agreement on how smoking affects the composition of gut microorganisms. In this study, the relationship between smoking status and gut microbial composition was investigated by performing 16S rRNA gene amplicon sequencing analysis of stool samples from 80 healthy Chinese adults. The results showed that smoking did not cause significant changes to the composition and microbial functional pathways of the gut microbiota. However, smoking altered the relative abundance of several specific taxa, where *Phascolarctobacterium* and *Fusobacterium* increased and *Dialister* decreased. Notably, our analysis revealed that smoking introduced more microbial interactions to the interaction network and decreased its modularity. Overall, this study provides new insights into the association between smoking status and the gut microbiota.

1. Introduction

Cigarette smoking is one of the most important public health issues in the world today. Despite a downward trend in the number of individuals who smoke, approximately 1.1 billion people are still current smokers, with China having the highest number, accounting for ~30 % globally [1,2]. Smoking is the leading cause of preventable deaths globally and a risk factor for many diseases, such as cardiovascular disease, chronic obstructive pulmonary disease, and cancer [3,4]. Therefore, there is growing public concern about the health effects of smoking.

The gut flora is a general term for the diverse microbial communities that parasitize the host intestine to maintain microecological balance. The human gut contains approximately 10 trillion bacteria, which play important roles in intestinal metabolism, immunity, inflammation, and neural signaling regulation through dynamic interactions with their host [5]. Dysbiosis of the intestinal flora is also associated with the development and progression of several diseases, such as cardiovascular disease, central nervous systemic disease, and cancer [6]. The composition of the gut microbiota differs in different human populations, which may be related to the genetic background, dietary habits, and living environment of the host [7]. Therefore, the gut microbial changes observed in specific populations may not be generalizable.

Cigarette smoke is a source of numerous toxicants, the components of which are dispersed in the gut after being inhaled and can perturb the intestinal microbial ecology via antibiotic effects, oxygen deprivation, or other potential mechanisms [8]. Although recent research suggests that cigarette smoke may alter the microbiota, the findings are not entirely consistent [9–13], which may be due to differences in the populations, sample sizes, and experimental methods used among the various studies. Therefore, the association between cigarette smoking and the gut microbiota remains unclear. Because there are not many studies on the effects of smoking on the gut microbiota of the Chinese population, this study was carried out to systematically compare the difference in gut microbial composition between current and never smokers in a Chinese cohort. The aim of the study was to gain new insights into the relationship between smoking and the gut microbiota.

2. Materials and methods

2.1. Study population

This study was approved by the Ethics Committee of the Binzhou Medical University Hospital (No: 2023-LW-14). All recruited individuals provided written informed consent to participate in the study. Individuals aged 20–65 years were recruited in 2023. The study participants submitted stool samples and completed a questionnaire on their demographics and lifestyle characteristics. Participants with missing or unknown demographic (age, race, or sex), body mass index (BMI), or

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smoking status data were excluded from the study. Finally, the stool samples of 80 participants who confirmed no use of antibiotics in the 2 weeks prior to sample provision were selected for further microbiota sequencing.

2.2. Fecal microbiota assay

The stool samples provided by all study participants were preserved in 95 % ethanol prior to being transferred to our biorepository within one day and stored at −80 ˚C before sequencing. DNA was extracted using the Stool Genomic DNA Extraction Kit (Beijing Solarbio Science & Technology Co., Ltd, Beijing, China) following the manufacturer's protocol. The V3-V4 region of the 16S rRNA gene was PCR amplified with the 341F/806R (CCT AYG GGR BGC ASC AG/GGA CTA CNN GGG TAT CTA AT) primer pair containing common adapter sequences and 6 bp barcodes. Next, Illumina flow cell adapters were added in a secondary PCR with 10 cycles of amplification. The PCR products were visualized using nucleic acid gel electrophoresis, purified using a gel extraction kit (CWBIO, Taizhou, China), and quantified using Qubit. Pooled amplicon libraries were sequenced using the 250 bp paired-end sequencing method on an Illumina NovaSeq system.

2.3. Bioinformatics analysis

The sequence reads were processed using the EasyAmplicon pipeline [14]. In brief, after the sequence reads had been demultiplexed, the paired-end reads were quality filtered and joined. Preprocessed sequences were clustered into amplicon sequence variants (ASVs) using USEARCH (v11.0.667). ASVs with a total abundance of less than 8 were excluded. The ASVs were taxonomically classified using the RDP database (v18) with a confidence liminal value of 80 %. To evaluate microbial richness and diversity, Chao1, Shannon, and Simpson indices were calculated using the "vegan" package in R (v4.1.1, R Foundation for Statistical Computing, Vienna, Austria). The relationship between smoking status (never smokers and current smokers) and the overall gut microbial community structure was assessed using Bray–Curtis, unweighted UniFrac distance, and weighted UniFrac distance analyses. The distances were computed using USEARCH (v11.0.667). Bray–Curtis distance-based principal coordinate analysis (PCoA) was conducted using Wekemo BioinCloud (https://www.bioincloud.tech). The linear discriminant analysis effect size (LEfSe) approach was used to determine bacterial taxa (biomarkers) whose abundance levels were significantly different between current and never smokers. The LEfSe analysis was conducted using online tools with default settings (http://huttenhower.sph.harvard.edu/galaxy/) [15]. Additionally, the machine learning approach in USEARCH (v11.0.667) was also used to identify ASVs with differences between the current and never smoker groups. Based on the ASV profiles in each group, the Spearman correlation coefficients among the ASVs were calculated using the "psych" package in R (v4.1.1), and the relations with coefficients of less than −0.6 or greater than 0.6 (q < 0.05, BH) were kept. Then, the co-occurrence networks were plotted and analyzed using Gephi (v0.9.2) [16]. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2) was used to infer functional shifts in the microbiota of the current smokers relative to that of the never smokers [17]. The Wilcoxon rank-sum test was used to compute the differences in the abundance of KEGG pathways. Differences with false discovery rate (FDR)-adjusted q-values of less than 0.05 were considered to be significant.

3. Results

3.1. Study cohort

In total, 80 individuals were included in the study. The participants were grouped as current smokers (n = 33) or never smokers (n = 47) according to their determined smoking status (Table 1). The current smokers had 25 years of smoking experience on average and consumed an average of 15 cigarettes per day (Table 1). Overall, they were nine years older than the never smokers (p = 0.038, Wilcoxon test), mostly males, and more likely to drink alcohol. There were no significant differences in BMI values between the individuals in the two groups (p = 0.161, Wilcoxon test) (Table 1).

3.2. No significant differences in the diversity of the fecal microbiotas were observed between the current and never smokers

The ASV number and Chao1, Shannon, and Simpson indices were calculated to determine the alpha diversity of the gut microbiota in the two participant groups. Although the current smokers had a slightly higher number of observed ASVs and a higher Chao1 index than the never smokers, the difference was not statistically significant (Wilcoxon test, p = 0.26 and 0.31, respectively) (Fig. 1A, 1B). Moreover, the current smokers had slightly smaller Shannon and Simpson indices, but the differences with the values for the never smokers were also not statistically significant (Wilcoxon test, p = 0.95 and 0.68, respectively) (Fig. 1C, 1D). These results indicated that there were no significant differences in the alpha diversity of the microbiotas between the current and never smokers.

To gain insights into the possible differences in microbial composition between the current and never smokers, we used Bray–Curtis distances to characterize the variations in species composition between the fecal samples. As shown in the PCoA plot (Fig. 1G), the overlapping distribution of samples between the current smokers (green dots) and never smokers (red dots) indicated no obvious differences in distribution between the two groups. Similarly, no significant differences were noted from the ANOSIM analysis (R = 0.009, p = 0.318) (Fig. 1E, 1F). Additionally, ANOSIM and PERMANOVA analyses based on unweighted and weighted UniFrac distances also showed no significant difference in the overall composition of the gut microbiota between the two groups (p>0.05) (Table S1). Therefore, these results indicated that there were no clear differences in microbial community composition between the current and never smokers in our study population.

Table 1
Overall participants' background.

	NS n = 47	CS n = 33	Overall cohort
Smoking history (mean ± SD)	0 ± 0	25±12	
Smoking amount (cigarettes/day, mean ± SD)	0 ± 0	15 ± 9	
Age (mean ± SD)	43±18	52 ± 16	47 ± 18
Female, n(%)	23 (49 %)	3 (9 %)	26 (33 %)
BMI (mean ± SD)	23.5 ± 3.9	25.8 ± 6.7	24.5 ± 5.4
Drinking status			
Non-drinker, n(%)	34 (72 %)	12 (36 %)	46 (58 %)
Former drinker, n(%)	9 (19 %)	13 (39 %)	22 (28 %)
Current drinker, n(%)	4 (9 %)	8 (24 %)	12 (15 %)

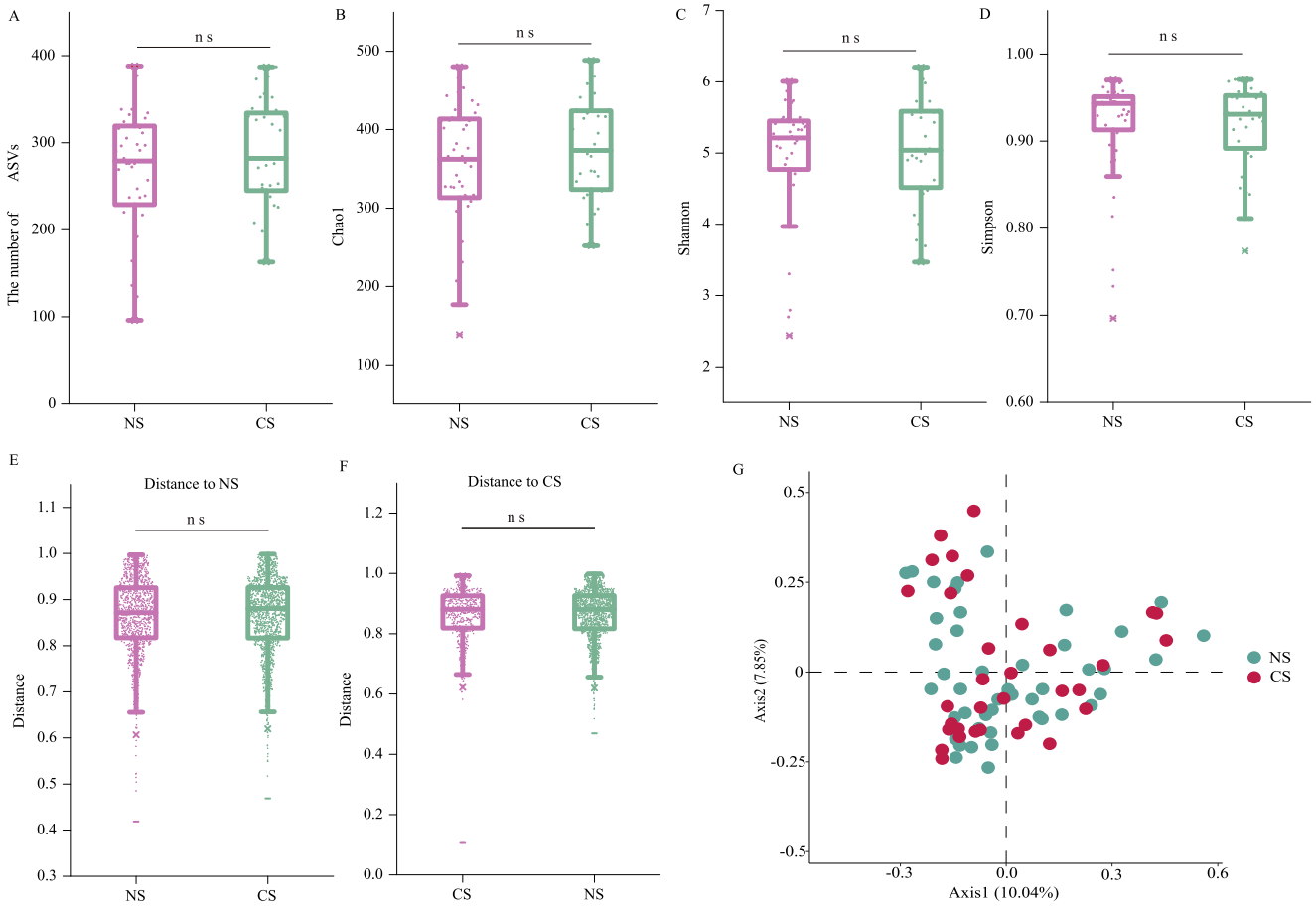


Fig. 1. Comparison of the gut microbiota in the current smokers (CS) and never smokers (NS). (A) Number of amplicon sequence variants (ASVs). (B) Chao1 index. (C) Shannon index. (D) Simpson index. (E) Bray–Curtis distance-based analysis of similarities. (F) Bray–Curtis distance-based analysis of similarities, distance to NS. (G) Bray–Curtis distance-based principal coordinate analysis, distance to CS.

Taxonomic classification revealed that the *Firmicutes* and *Bacteroidetes* were the most abundant phyla in both the current and never smokers (Fig. 2A). The phyla *Proteobacteria* and *Actinobacteria* were also in high abundance in both groups (Fig. 2A). At the genus level, *Prevotella, Faecalibacterium, Phocaeicola, Bacteroides, Pseudescherichia*, and *Bifidobacterium* were the most abundant taxa in both the current and never smokers (Fig. 2B).

3.3. The abundance of specific taxa differed between the current and never smokers

We next sought to investigate whether there were differences in the abundance of specific taxa between the two participant groups. LefSe analysis revealed that a few taxa were enriched in the current smokers, including *Phascolarctobacterium* (o: *Acidaminococcales*; f: *Acidaminococcaceae*; g: *Phascolarctobacterium*) and *Fusobacterium* (p: *Fusobacteria*; c: *Fusobacteriia*; o: *Fusobacteriales*; f: *Fusobacteriaceae*; g: *Fusobacterium*), whereas members of *Dialister* (o: *Veillonellales*; f: *Veillonellaceae*; g: *Dialister*) were decreased in this group (Fig. 3A and 3B). Similarly, the machine learning method revealed that *Phascolarctobacterium* (ASV_1792) and *Fusobacterium* (ASV_14) were enriched, and *Dialister* (ASV_11) was decreased in the current smokers (Fig. 3C, Table S2). The machine learning method also revealed that the *Bacteroidaceae* (ASV_148, 462, 110, 137, 1532, 1962), *Clostridiales* (ASV_1291, 601), *Negativibacillus* (ASV_250), and *Allisonella* (ASV_1665) were enriched in the current smokers (Fig. 3C, red dots, Table S2), whereas the *Faecalibacterium* (ASV_4) and *Bifidobacterium* (ASV_162) were depleted (Fig. 3C, green dots, Table S2).

3.4. Co-occurrence network analysis suggested more frequent interactions but lower modularity in the network in the current smokers

Based on their relative abundance, we calculated the Spearman correlation coefficients between ASVs for the current and never smokers. The analyses revealed a much higher number of positive correlations between ASVs (R>0.6, q<0.05) in the current smokers than in the never smokers (6905 vs. 3753) and a few negative correlations (R<−0.6, q<0.05) for both groups (29 in current smokers and 2 in never smokers) (Figure S1). Considering that the total number of ASVs in the current smokers was slightly higher than that in the never smokers (1105 vs. 982), the above result indicated that the gut bacteria in the current smokers had more and probably stronger interactions than those in the never smokers. Further co-occurrence network analysis was performed based on the above correlations (Fig. 4A, 4B). Consistent with the findings of a higher number of correlations in the current smokers, the network analysis revealed higher graph densities and average degrees in this smoker group. The analysis also revealed that the modularity index, clustering coefficient, and average path length were lower for the current smokers (Fig. 4B), indicating a lower level of modularity of the microbial interaction network in this group.

Thus, the above results indicated that the microbial interactions in current smokers are different from those in never smokers. Compared

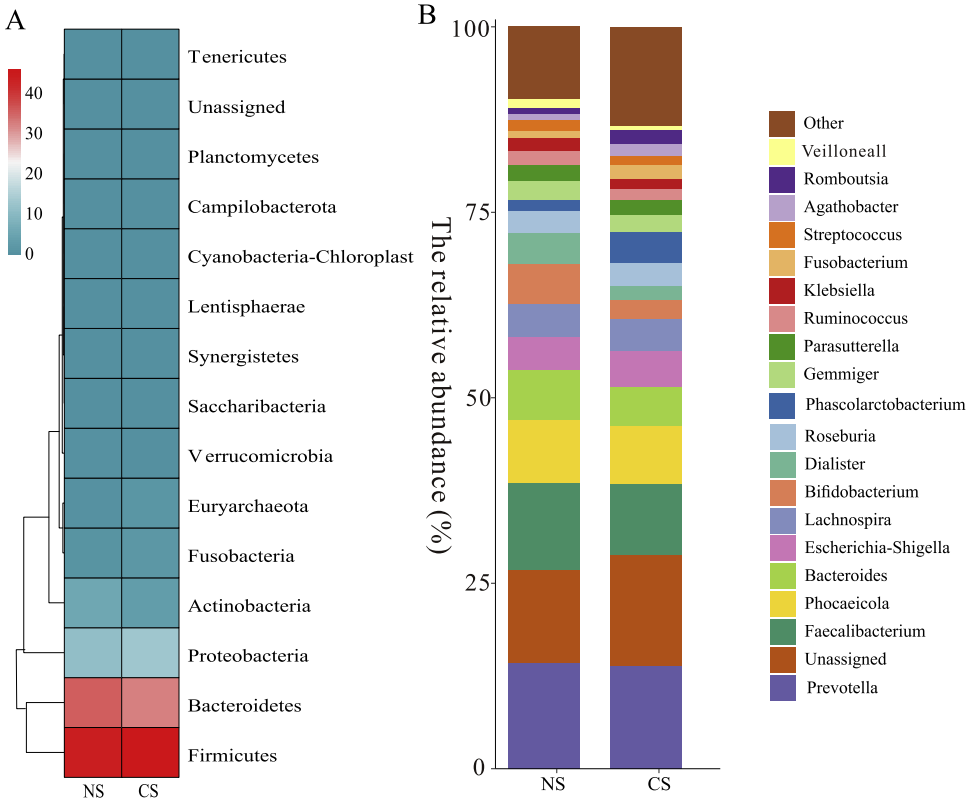


Fig. 2. (A) Comparison of the microbial compositions at the phylum level. (B) Comparison of the microbial compositions at the genus level.

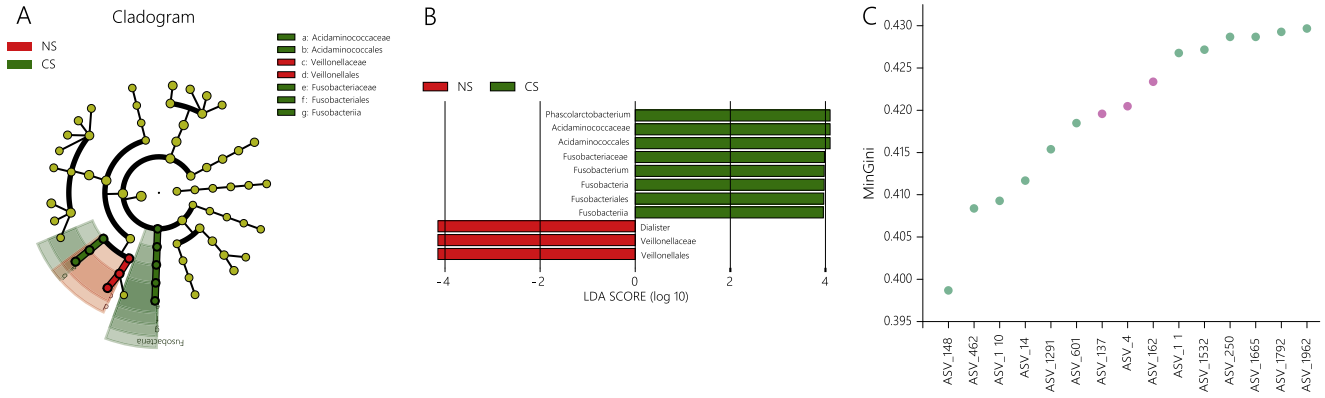


Fig. 3. Taxa with different abundances in the current smokers compared with those in the never smokers. (A, B) Taxa associated with smoking status identified with the linear discriminant analysis effect size (LEfSe) method. Green dots represent taxa that are enriched in the current smokers (CS), and red dots represent taxa that are enriched in the never smokers (NS). (C) Fifteen amplicon sequence variants (ASVs) that differed most between the CS and NS groups identified with the machine learning method. A smaller minGini value indicates a greater difference in the ASV distribution between the two groups.

with those in never smokers, the interactions between microbial taxa in current smokers are more frequent and probably stronger, and the interaction network has lower modularity.

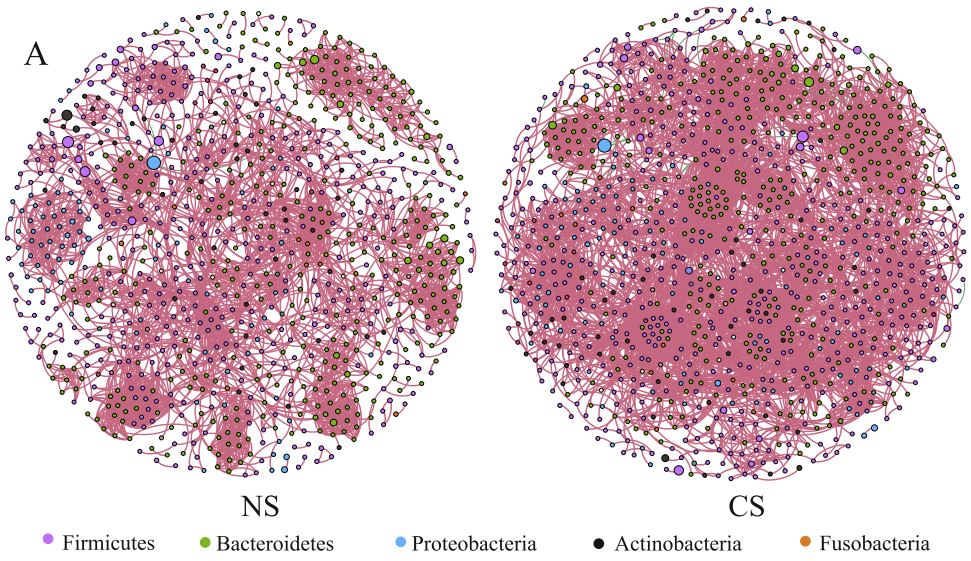
3.5. No significant difference in the functional pathways of the fecal microbiotas was observed between the current and never smokers

To investigate the functions of the gut microbiota under different smoking statuses, PICRUSt2 analysis based on inferred metagenomes was performed. In total, 320 KEGG pathways were identified (Table S3), with those related to the metabolism of amino acids, carbohydrates, and vitamins and cofactors being abundant in both the current and never smokers (Fig. 5). Generally, the abundance of each pathway was similar for the two groups. Although 20 pathways (e.g., those for stilbenoid, diarylheptanoid, gingerol, and flavonoid biosynthesis and amino and nucleotide sugar metabolism) had p-values lower than 0.05, the differences between the groups were not statistically significant based on the FDR correction (q>0.05, BH) (Table S3).

4. Discussion

4.1. Diversity of the gut microbiota

Approximately 1.1 billion people worldwide use tobacco products [1]. In recent years, the incidence of smoking-related diseases, including cardiovascular disease, chronic obstructive pulmonary disease, and various types of cancer, has increased drastically [3,4], indicating the potential harm of smoking in the development of human disease. Additionally, studies have found that cigarette smoking or exposure to secondhand smoke is associated with the colonization of potentially pathogenic



B

Topological properties of above cooccurrence networks

Group	Node	Edge	Modularity index	Clustering coefficient	Graph density	Average degree	Average path length
CS	1105	6934	0.781	0.481	0.011	12.55	5.045
NS	982	3755	0.873	0.525	0.008	7.655	7.714

Fig. 4. (A) Co-occurrence networks of gut microbes in the current smokers (CS) and never smokers (NS). Each node in the network represents an amplicon sequence variant (ASV). The color of the node indicates the phylum and the size reflects its abundance. Edge linking nodes represent established correlations between ASVs. Red edges represent positive correlations, and blue edges represent negative correlations. (B) Topological properties of the co-occurrence networks in the CS and NS groups.

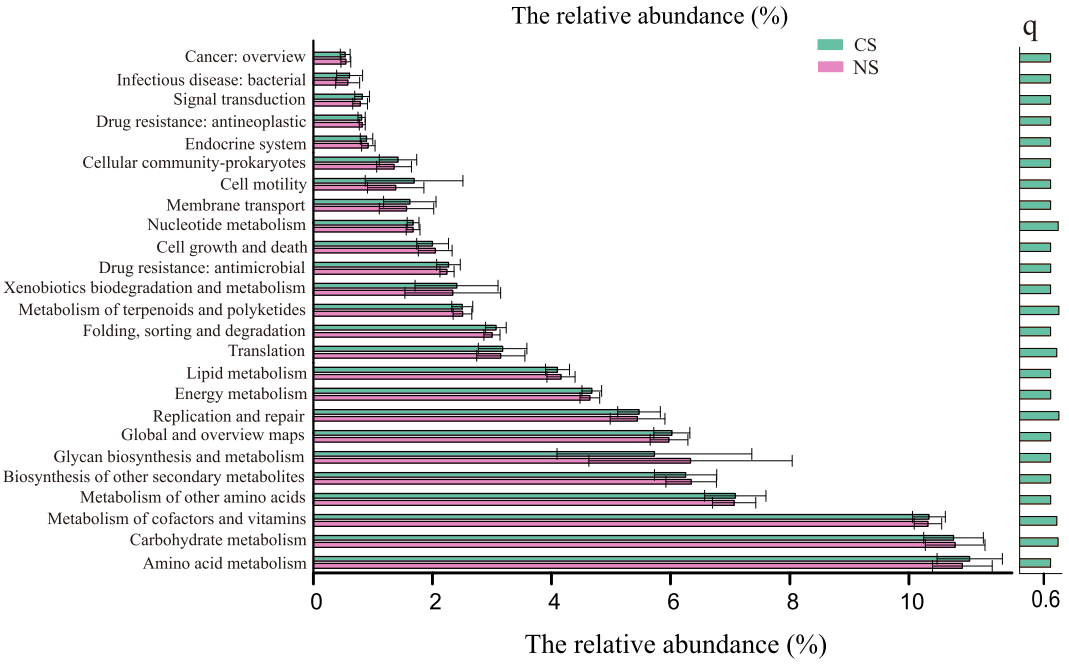


Fig. 5. Differential analysis of microbial community functions between the current smokers (CS) and never smokers (NS) (KEGG pathways, level 2). The statistical significance value (FDR q-value, BH) is shown on the right.
microbes in current smokers (CS) and never smokers (NS).

bacteria [18]. However, studies on the effects of cigarette smoking on the gut microbiota are limited, and the conclusions vary from study to study. To gain insights into the relationship between smoking and the gut microbiota, we collected fecal samples from 80 individuals for 16S rRNA gene amplicon sequencing to search for differences in the gut microbial taxa between current and never smokers.

A few studies have found that cigarette smoking causes a decrease in the alpha diversity and a significant change in the beta diversity of the gut microbiota [9,11,19]. Other studies have reported that there is no significant difference in the alpha and beta diversity of the gut microbiota between current and never smokers [20,21], which is consistent with the findings in our present study. The gut microbiota is influenced

by a variety of factors, such as the geographical location, ethnicity, and dietary habits of the human host [7]. Studies have also produced inconsistent or even conflicting findings of associated gut flora for the same type of disease [22,23]. Recently, He et al. [22] found that regional factors have a significantly greater effect on the gut microbiota than other factors and proposed that such regional-based variations limit the application of healthy gut microbiota reference ranges and disease models. Therefore, regional and other factors may contribute to the inconsistent conclusions between studies on the smoking–microbe relationship. Our present study provides new data on the association between gut microbiota and cigarette smoking.

Similarly, conclusions on the impacts of age, sex, and drinking status on gut microbes are also controversial. For example, Zhang et al. [24] found the existence of sex- and age-related trajectories of the human gut microbiota that are shared between populations of different ethnicities. Chen et al. [20] found that the host BMI, race, sex, and alcohol use were significantly associated with the microbial beta diversity. However, Cuesta-Zuluaga et al. [25] revealed that the influence of age and sex on gut microbes varied across populations, and there were minimal associations between these two factors and gut biodiversity in their Chinese cohort. Another study showed that the gut microbiota had a highly positive correlation with the host age but no significant correlation with the host sex [26]. Because the gut microbial composition is influenced by various factors, precisely quantifying the impact of a particular factor on the microbes is difficult, and more efforts to control the variables are required.

4.2. Veillonellaceae and Fusobacterium

Smoking may alter the abundance of specific taxa. A study of a healthy Bangladesh population found that smoking led to an increase in the relative abundance of the *Erysipelotrichi* and *Catenibacterium* lineage in the gut microbiota [27]. These taxa were not noted in our study. Another study conducted on the New York University Food and microbiota Longitudinal Investigation Study Cohort found an increase in the *Prevotella* and *Veillonellaceae* taxa and a decrease in the *Lachnospira* and *Tenericutes* in the gut microbiota of smokers [28]. These findings are partially consistent with those of our study, which revealed that *Dialister*, a genus within the family *Veillonellaceae*, was depleted in the current smokers. Our study also revealed that *Fusobacterium* was enriched in the current smokers. *Fusobacterium* is an opportunistic pathogen associated with diseases such as ulcerative colitis, Crohn's disease, and colorectal cancer [29,30]. Similarly, Shanahan et al. [31] found *Fusobacterium* to be enriched in the gut microbiota of current smokers.

4.3. Phascolarctobacterium and Dialister

Species of the genus *Phascolarctobacterium* colonize the human gut in large numbers and preferentially utilize succinate [32]. Those of the bacterial genus *Dialister* are also common in the human intestinal flora, metabolizing carbohydrates and producing succinic acid, acetic acid, and propionic acid [33]. One study found that a high abundance of *Phascolarctobacterium* was significantly associated with weight loss success, whereas a high abundance of *Dialister* was associated with weight loss failure [34]. Spindler et al. found that current smokers tended to weigh less than never smokers and were likely to gain weight after quitting smoking [35]. The weight gain in quitters may be associated with changes in the gut microbiota [35]. Interestingly, our study showed that the genus *Phascolarctobacterium* was enriched in the current smokers, whereas *Dialister* was depleted in this group. In our cohort, the current and never smokers had similar weights (BMI). Therefore, it is unlikely that smoking alters the abundance of *Phascolarctobacterium* and *Dialister* by changing the body weight. Instead, it is more likely that smoking alters the abundance of these two genera through other unknown mechanisms that may subsequently alter the body weights of some smokers. The smoking–gut microbiota–host body weight relationship and the mechanisms underlying these associations need further study.

4.4. Bifidobacterium, Faecalibacterium, and Bacteroides

The relative abundances of important probiotics such as *Bifidobacterium* and *Faecalibacterium* were reduced in the current smokers in our study (Fig. 2C, Table S2). Some *Bifidobacterium* and *Faecalibacterium* species have anti-inflammatory effects, maintain bacterial enzyme activity, and protect the digestive system from intestinal pathogens [36,37]. In our study, many ASVs of *Bacteroides* were enriched in the current smokers (Fig. 2C, Table S2), which is consistent with the findings of previous studies that the relative abundance of this genus increases significantly in smokers [21,38,39]. Members of the *Bacteroidetes*, the main flora of the intestine, are involved in many important metabolic activities in the gut, including the fermentation of carbohydrates, utilization of nitrogenous substances, and biotransformation of steroids [40]. Recent studies have even found that *Bacteroides xylanisolvens* is involved in the degradation of nicotine, suggesting that members of the *Bacteroidetes* play important roles in maintaining health and mitigating the harmful effects of cigarette smoking [41].

4.5. Microbial interactions in the community

This study revealed that smoking extensively alters the number and probably the strength of community interactions between the gut microbiota (Fig. 3). No other studies have reported changing patterns of interactions within the gut microbiota of smokers. However, one study on peri-implant microbial communities reported that smoking leads to fewer microbial interactions, a finding contrary to our results [42]. As there are few published research papers on how smoking affects the interaction between gut microbes, further studies are needed to elucidate the underlying mechanisms involved.

Conclusion

In summary, our study revealed that smoking did not cause significant differences in the alpha and beta diversity of the gut microbiota in our cohort. However, smoking affected the relative abundance of specific taxa, where *Phascolarctobacterium* and *Fusobacterium* increased and *Dialister* decreased. Moreover, smoking affected the structure of the co-occurrence network, leading to more interactions between taxa and a decrease in the network modularity. The results of this study provide new insights into the association between smoking and the gut microbiota.

Data Availability Statement

The raw sequence data have been deposited at the Beijing Institute of Genomics Data Center (BIGD) under the genome sequence archive (GSA) number CRA011053 (http://bigd.big.ac.cn/gsa/s/xWfbv74D).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT Authorship Contribution Statement

Zhouhai Zhu: Writing – original draft, Methodology. **Meng Wang:** Writing – original draft, Methodology. **Ying Guan:** Software, Project administration. **Meng Li:** Validation, Supervision. **Qiyuan Peng:** Investigation, Formal analysis. **Ning Zheng:** Project administration, Formal analysis. **Wenbin Ma:** Writing – review & editing, Funding acquisition, Conceptualization.

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Supplementary Materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.engmic.2024.100138.

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