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Individuals with abnormal cardiometabolic statuses are more vulnerable to smoking-induced alterations of the gut microbiome in a Colombian population undergoing Westernization

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SUMMARY The "Western" diet and lifestyle is of increasing scientific interest due to its rising global prevalence and potential implications for human health. It is associated with a decline in gut microbiome diversity and an abnormal cardiometabolic status, which contribute to inflammatory responses and the development of non-communicable diseases. Additionally, smoking has been implicated in increased morbidity and mortality from cardiovascular diseases and metabolic disorders. However, interactions between smoking, cardiometabolic status, and the gut microbiome have not been previously characterized in the context of Westernization. In this study, we investigated the impact of smoking on the gut microbiome across westernized Colombian individuals with either healthy or abnormal cardiometabolic statuses which were collected during a study performed by *Cuesta-Zuluaga et al*. For those with an abnormal cardiometabolic status, smoking was associated with reduced microbial species richness, particularly distinct in 8 core microbiome members, including the commensal genera of *Bifidobacteria*, *Christensenellaceae,* and *Blautia*. For westernized individuals with either a healthy or abnormal cardiometabolic status, smoking was associated with a reduced relative abundance of bacterial genera. However, the number of genera experiencing a decrease in relative abundance was greater in individuals with abnormal cardiometabolic statuses. The decrease in relative abundance was represented primarily in the *Firmicutes* phylum. Furthermore, abnormal cardiometabolic status was associated with potentially harmful bacterial species, such as *Desulfovibrio piger* and *Bacteroides ovatus*, which was significantly differentially abundant in smokers and are also prevalent in inflammatory bowel disease patients. Our findings indicate that individuals with abnormal cardiometabolic statuses are more susceptible to smoking-induced changes of their gut microbiomes.

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INTRODUCTION

Factors affecting the gut microbiome include one's dietary intake, level of activity, medication usage, sex, geographical location, and other lifestyle-related variables (1-5). Currently, a global trend of "Westernization" is influencing a shift of the less industrialized societies towards the industrialized, North American, and European way of life. In regions experiencing this transition, the factors affecting the microbiome are consequently becoming Westernized, which is concerning, as the Western-associated microbiome is implicated in dysbiosis and non-communicable diseases (2, 6). Therefore, conducting microbiome studies

in these communities is important as it allows us to address human health in the context of cultural globalization.

Westernization is often associated with dietary changes that decrease microbiome diversity and induce pathological alterations to the gut microenvironment (2, 6, 7). The Western diet is characterized by an excessive consumption of processed and/or high energy density foods, and an insufficient intake of foods rich in fiber, fruits, and vegetables (2, 7, 8, 9). Processed and high energy density foods are often produced using refined, modified, and/or synthetic ingredients (7, 10). These dietary changes often occur alongside lifestyle changes, the most important of which is decrease in physical activity (12). A sedentary lifestyle is a major risk factor in the development of non-communicable diseases, such as coronary heart disease, type two diabetes, and breast cancer (4, 5). Physical activity is shown to shift the microbiome's diversity and composition towards a healthier state, although this effect is also influenced by the host's physiological status and dietary intake (4).

Smoking is another risk factor for the development of non-communicable diseases, and previous studies have identified differences in the microbiome composition of smokers and non-smokers (13-15). Smoking leads to the development of chronic respiratory diseases, cardiovascular diseases (CVDs), and cancer (15). CVDs promote inflammatory conditions in the body, which worsen metabolic health (15). Smoking has been associated with increased insulin resistance, atherosclerosis, dyslipidemia, and diabetes, demonstrating a link between smoking and metabolism (15). Additionally, smoking reduces microbiome diversity, and induces compositional alterations like those observed in the contexts of irritable bowel disease and obesity (13, 16). The potential mechanisms behind smoking-induced gut microbiome alterations are diverse, and include disruptions to immune homeostasis, increased biofilm formation, the introduction of cigarette-borne bacteria, and heightened oxygen tension (13). Smoking-induced alterations to cardiometabolic health and the microbiome align with changes attributed to Westernization, therefore it is critical to further characterize associations between these factors and to elucidate their intersectional roles in human health (2, 4-7, 11, 13-15).

The metadata for our study was collected by *Cuesta-Zuluaga et al.*, whose research group aimed to investigate the microbiome of Colombians undergoing Westernization. The methodological approach used differs from typical microbiome studies, because subjects were first classified by microbial composition rather than by clinical conditions (2). They then used the microbial composition data to investigate correlations with host health-associated variables. It was observed that traditional, Colombian diet-associated microbial taxa, such as *Prevotella* and *Treponema,* were present alongside Western-diet associated taxa, such as *Bacteroides* and *Escherichia.* These findings suggested a gradual change in microbiome composition, which may be indicative of the increasing influence of the Western diet and lifestyle (2). Individuals in the study were characterized as cardio-metabolically healthy or abnormal using clinical variables measured in blood serum, which can act as markers for non-communicable diseases (2). These clinical metrics represent an individual's lipid profile, glucose metabolism, inflammatory C-reactive protein, and blood insulin, providing a comprehensive profile of cardiometabolic health. *Cuesta-Zuluaga et al.* collected data for multiple host health-related variables, including smoker and non-smoker identification.

In the current study, we aim to identify correlations between the gut microbiome, cardiometabolic status, and smoking in a Colombian population who are experiencing Westernization. Using alpha diversity metrics, we observed significant decreases in the microbial species richness of smokers relative to non-smokers in individuals with abnormal cardiometabolic statuses. Core microbiome analysis found that smokers and non-smokers with healthy cardiometabolic statuses shared a greater number of core microbial taxa relative to those with abnormal cardiometabolic statuses. Additionally, we identified a distinct decrease in 8 core microbiome members in smokers with abnormal cardiometabolic statuses. Using differential abundance analysis, we found that smoking was associated with a decrease in the relative abundance of amplicon sequence variants (ASVs) across individuals of both cardiometabolic statuses. However, a greater number of ASVs were decreased in relative abundance in individuals with abnormal cardiometabolic statuses. Our findings illustrate an

enhanced susceptibility to detrimental smoking-induced microbiome changes in individuals with abnormal cardiometabolic statuses, experiencing Westernization.

METHODS AND MATERIALS

Dataset and metadata. The original dataset by *Cuesta-Zuluga et al.* was generated for a cross-sectional study conducted in 2014, to characterize the gut microbiota of 441 Colombian adults (2). Fecal samples were collected from participants and microbial DNA was extracted via the QIAamp DNA Stool Mini Kit (2). The bacterial 16S rRNA V4 region was amplified using primers F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′) and sequenced on the Illumina MiSeq sequencing platform (2). Participant age range varied between 18-62 years old, and excluded individuals who were underweight, pregnant, or took antibiotic/antiparasitic medication 3 months prior to sample collection (2). People diagnosed with neurodegenerative diseases, cancers, or gastrointestinal diseases were also excluded from the study (2). Participants represented different cities of origin, ages, and sexes at similar proportions (2). The study metadata contained information on cardiometabolic status, smoking status, age, sex, BMI, geographical location, whole blood analysis results, and diet, among other factors (2). The dataset is available through SRA-NCBI under BioProject PRJNA417579. Further information about sample collection and experimental methods can be found in the original study (2).

Data processing using the QIIME2 pipeline. The sequence data were imported into and demultiplexed using Quantitative Insights into Microbial Ecology Version 2 (QIIME2, version 2023.5) (17). Sequence quality control was performed using the Divisive Amplicon Denoising Algorithm 2 (DADA2) pipeline via the Conda environment (18). The ASVs were determined using a truncation length of 225 nucleotides, which maintained a minimum Phred quality score of 30 during denoising and clustering steps. To assign taxonomy to the ASVs, we classified the data using the SILVA 138-99 database on QIIME2 (19). Mitochondrial and chloroplast sequences were subsequently filtered out of the resulting feature table using QIIME2 quality control scripts. In total, there were 441 samples, with 269 and 172 samples being classified as having healthy and abnormal cardiometabolic statuses, respectively. After filtering based on cardiometabolic status, there were 239 and 30 samples that were non-smokers and smokers, respectively, in the cardiometabolically healthy group; and 144 and 28 non-smokers and smokers, respectively, in the cardiometabolically abnormal group. Samples were alpha-rarefied for downstream analyses at a depth of 20,291 sequences per sample, with 8,217,855 (50.2%) ASVs retained afterwards.

Alpha and beta diversity analysis. The QIIME2-processed data were imported into Rstudio (version 4.1.1) for downstream analyses (20). The data were combined into one phyloseq object using the phyloseq package (21) and filtered to exclude samples with fewer than 100 reads and ASVs with low-abundance reads (<5). The phyloseq object was then subsetted based on cardiometabolic status and subsequently grouped according to smoking status. Alpha diversity was assessed using the Observed, Chao1, and Shannon indexes with statistical significance determined by pairwise Kruskal-Wallis tests, conducted using the phyloseq package in R 4.2.2 (21). Beta diversity was assessed using Unweighted UniFrac distance analysis, with statistical significance determined via the multivariate adonis2 test (1000 permutations), also conducted in R using the vegan package (22). To visualize community composition differences, Principal Coordinate Analysis (PCoA) plots were generated using the ggplot2 package in R (23).

Core microbiome analysis. The core microbiome across all subsetted samples was analyzed using the resulting phyloseq object in R (20). The core microbiome was defined as the set of ASVs that were present in at least 50% of the samples (prevalence of 0.5), and with a detection threshold of 0.001, using the phyloseq package (21). To compare all four population subsets, a four-way venn diagram was generated using the ggVennDiagram package (24) in R, with data preparation and visualization conducted using the following R packages: vegan (22),

ggplot2 (23), dplyr (25), tidyr (26), ggpubr (27), microbiome (28), ape (29), tidyverse (30), and grid (31).

Differential relative microbial abundance analysis. Differential relative microbial abundance analysis was performed in R using the generated phyloseq object (20). The Wald test was used to calculate P-values, and differentially abundant microbial taxa were identified based on a significance threshold of adjusted P-value = 0.01 and an effect threshold of absolute $\log_2$ fold change > 2, using the DESeq2 package (32). Heatmaps and volcano plots were generated to visualize the results, using various R packages including ggplot2 (23), dplyr (25), tidyr (26), microbiome (28), ape (29), and tidyverse (30), which were also utilized for data processing.

RESULTS

Abnormal cardiometabolic status is correlated with reduced richness in smokers. To identify whether smoking affects the microbiome of cardiometabolically healthy and abnormal Colombians in terms of richness and evenness, we subsetted the samples based on cardiometabolic status and determined the Observed, Chao1 and Shannon alpha diversity metrics (Fig. 1). Smoking did not have a significant impact on the microbial richness of individuals with healthy cardiometabolic statuses (Fig. 1A). However, in smokers with abnormal cardiometabolic statuses there was a significant reduction in richness, particularly in rare ASVs, relative to non-smokers (Fig. 1B). For Shannon diversity, which accounts for richness and abundance, there was no significant difference between the microbiomes of smokers and non-smokers with either healthy or abnormal cardiometabolic statuses (Fig. 1). Therefore, smoking is associated with a reduction in richness, but not abundance, in individuals who have abnormal cardiometabolic statuses.

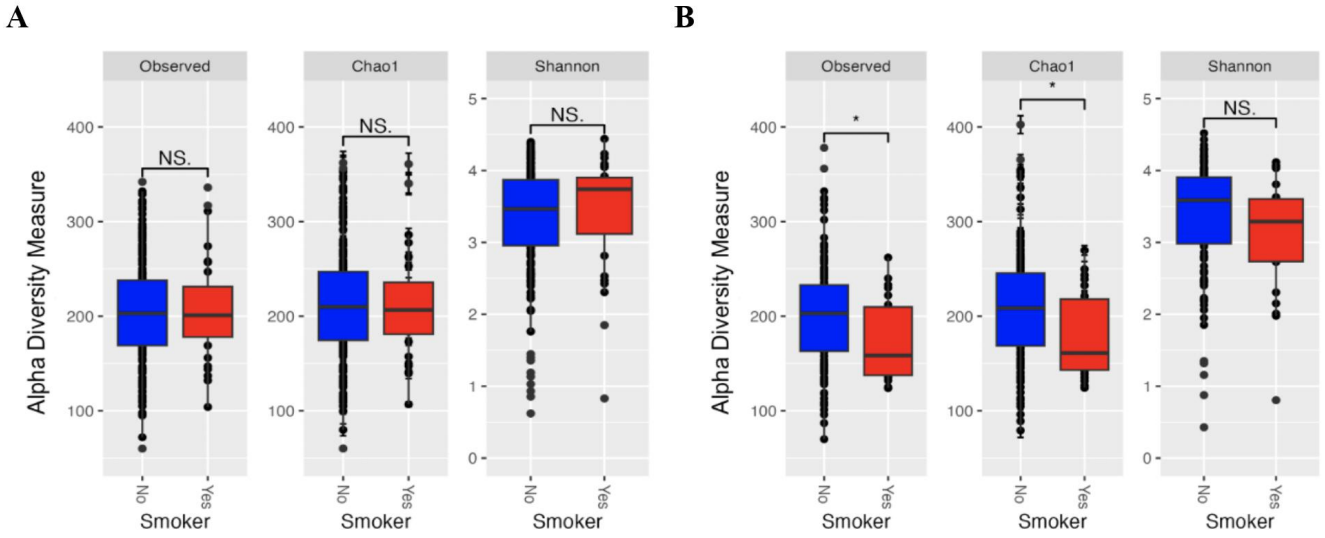
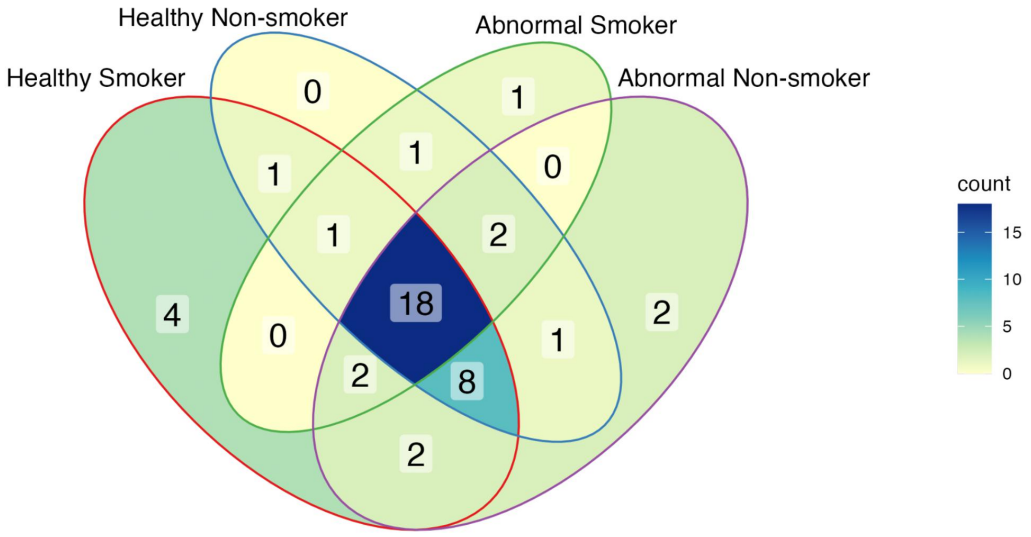


FIG. 1 Smokers with abnormal cardiometabolic statuses have a significant reduction in richness for the Observed and Chao1 metrics. Comparing microbial diversity between smokers and non-smokers based on cardiometabolic status using the alpha diversity metrics: Observed, Chao1 and Shannon. **(A)** Healthy cardiometabolic status. **(B)** Abnormal cardiometabolic status. Significance analyzed via Kruskal-Wallis test; *p <0.05.

Abnormal cardiometabolic status is associated with a smaller core microbiome compared to cardio metabolically healthy smokers and non-smokers. To determine the distribution of shared and unique ASVs amongst the groups of smoking and non-smoking Colombians with either healthy or abnormal cardiometabolic statuses, a core microbiome analysis was performed using R (Fig. 2). Smokers and non-smokers with healthy cardiometabolic statuses shared a greater proportion of their core microbiome, with 70% of their total ASVs being shared, compared to those with abnormal cardiometabolic statuses, who shared 58% (Fig. 2A). For individuals with healthy cardiometabolic statuses, smokers

A



B

Phylum	Genus
Actinobacteria	*Bifidobacterium*
Firmicutes	*Christensenellaceae_R-7_group*
Firmicutes	*Christensenellaceae_R-7_group*
Firmicutes	*Streptococcus*
Firmicutes	*Clostridium_sensu_stricto_1*
Firmicutes	*UCG-002*
Firmicutes	*UCG-002*
Firmicutes	*Blautia*

FIG. 2 Individuals with abnormal cardiometabolic statuses are susceptible to smoking-induced reductions in the number of core microbiome members. (A) Core microbiome analysis to determine shared and unique ASVs by cardiometabolic status and smoking. Using R, a core microbiome analysis was performed with a prevalence of 0.5 and a detection of 0.001. Count numbers indicate the quantity of ASVs belonging to each respective grouping. **(B)** The phyla and genera of the 8 core ASVs absent from the cardiometabolically abnormal

possessed a greater number of unique core microbiome members, with 8 unique ASVs, relative to 4 for non-smokers (Fig. 2A). In individuals with abnormal cardiometabolic statuses, the opposite trend was observed, where smokers had 3 unique core microbiome members versus 13 for non-smokers (Fig. 2A). The four groups possessed a shared microbiome of 18 core members (Fig. 2A). However, there were 8 core microbiome members absent from smokers with abnormal cardiometabolic statuses but shared by the other three

groups (Fig. 2A). From this, it appears that an abnormal cardiometabolic status leads to a susceptibility to smoking-associated reductions in the number of core microbiome ASVs. To further characterize this distinct reduction, we used R to identify the phyla and genera of these 8 ASVs. 7 of the 8 ASVs belonged to the *Firmicutes* phylum, with 1 belonging to *Actinobacteria*. Furthermore, the 8 ASVs belonged to 6 genera, including *Bifidobacterium, Blautia*, *Christensenellaceae R-7 group, Clostridium sensu stricto 1, Streptococcus,* and *UCG-002* (Fig. 2B).

Smoking is correlated with reduced relative abundances of bacterial species compared to non-Smokers, regardless of cardiometabolic status. To determine the differential expression of ASVs between smoking and non-smoking Colombians with either healthy or abnormal cardiometabolic statuses, a differential expression sequence analysis was performed using R at the genus level (Fig. 3 A-B). There was a significant reduction (p adj < 0.01) in differentially abundant genera in the smoking group compared to the non-smoking group for individuals of both healthy and abnormal cardiometabolic statuses (Fig. 3A). In the cardiometabolically healthy group, we identified 56 bacterial genera with a $\log_2$ fold change greater than 2 in the non-smokers group relative to the smokers. In the cardiometabolically abnormal group, we observed a greater reduction in relative abundances between smokers and non-smokers, with 101 bacterial genera enriched in non-smokers (Fig. 3B). This suggests

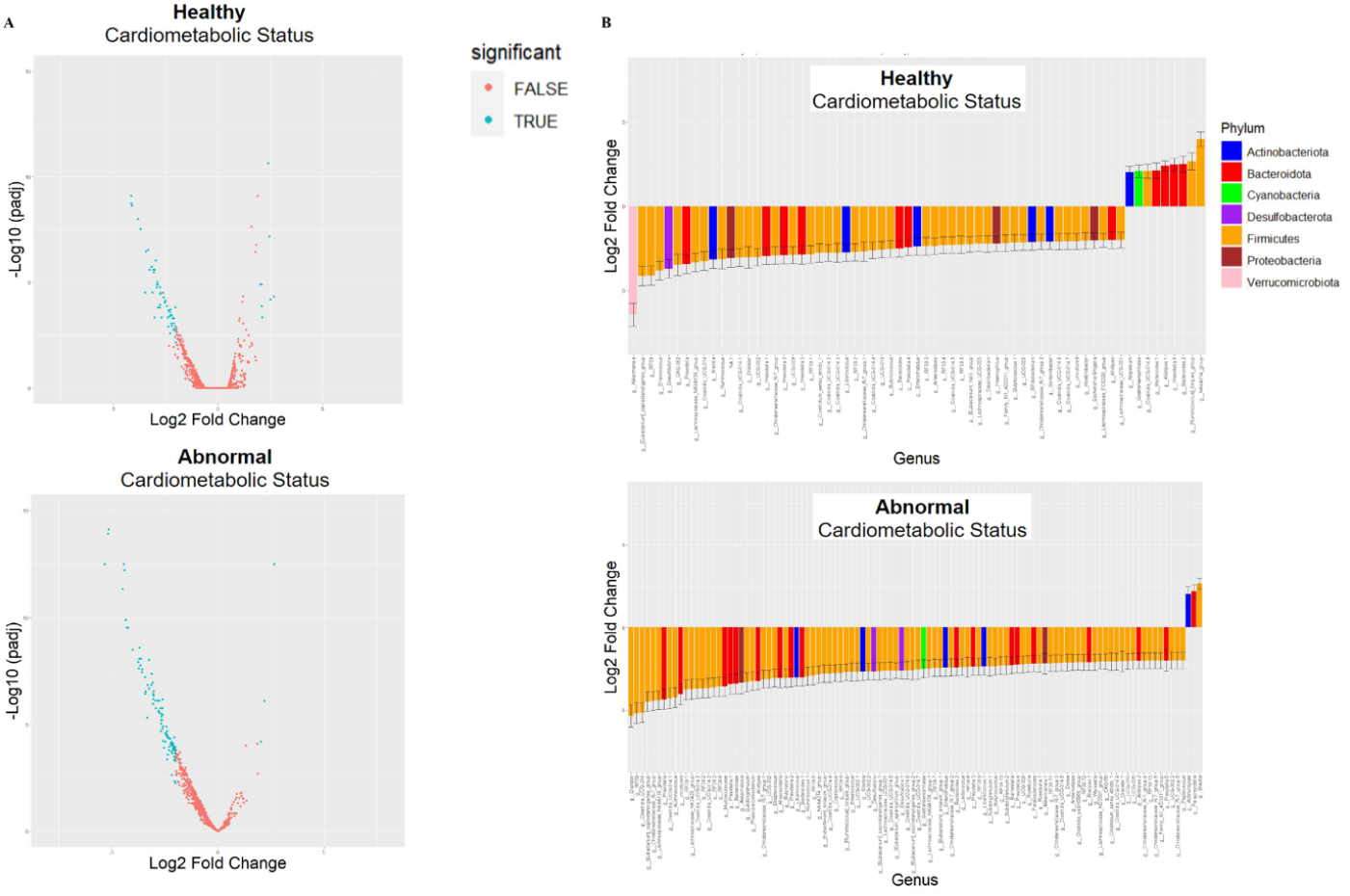


FIG. 3 Smokers display reduced differential abundances of gut bacteria relative to non-smokers in both cardiometabolic status groups. Comparing differential abundances of bacterial ASVs between smokers and non-smokers based on cardiometabolic status using DESeq2 analysis. The Wald test was used to calculate p-values. **(A)** Volcano plot. Blue dots: $|\text{Log}_2$ fold change$| > 2$ of smokers vs. non-smokers for each cardiometabolic status cohort. significant differential abundance, p adjusted = 0.01 **(B)** Taxa bar plot. Differential abundance of bacterial species based on Genus and shown in color according to phylum: $|\text{Log}_2$ fold change$| > 2$ of smokers vs non-smokers for each cardiometabolic status cohort. *All ASVs are significantly differentially abundant, p adjusted = 0.01.

that cardiometabolic status influences the severity of the relative smoking-associated reduction in bacterial species. Notably, 70% of the differentially reduced species in the healthy group and 75% in the abnormal cardiometabolic status group belonged to the *Firmicutes* phylum (Fig. 3B). To better characterize the changes in relative abundances, we identified the differentially expressed ASVs which were either shared or unique for each cardiometabolic status group. We found 26 shared ASVs, 40 ASVs unique to the cardiometabolically healthy pool, and 79 ASVs unique to the cardiometabollicaly abnormal pool (Table S1). For both groups, these ASVs belonged primarily to the *Firmicutes* phylum.

DISCUSSION

In this study, we aimed to investigate potential associations between smoking, cardiometabolic status, and gut microbiome composition in a Colombian population undergoing Westernization. Although previous studies have extensively characterized the isolated effects of smoking and cardiometabolic health on gut microbial diversity (2, 3, 11, 13-16), the interplay of their effects amidst the broader trend of Westernization has not been fully elucidated.

Our findings suggest that individuals who both smoke and have an abnormal cardiometabolic status display significant reductions in the richness of their gut microbiomes compared to non-smokers. This observed reduction in gut microbiome richness among individuals who smoke and have abnormal cardiometabolic status is consistent with the results of prior studies, where smoking and cardiometabolic disorders have been independently linked to reductions in gut microbial diversity (11, 13, 16, 33-35). Our results support the notion that smoking and cardiometabolic status can have a combined effect on the gut microbiome. Specifically, cardiometabolic disorders and their underlying risk factors may lead to a gut microbiome that is more susceptible to detrimental, smoking-induced alterations. The reduction in gut microbiome richness among smokers with abnormal cardiometabolic status is likely driven by a number of factors. Cardiometabolic disorders are associated with gut microbiome dysbiosis and an altered gut microenvironment, which can impair the growth and survival of potentially beneficial gut bacteria (34, 35). Similarly, smoking has been shown to alter the gut microenvironment, reducing the production of potentially beneficial, anti-inflammatory short-chain fatty acids (SCFAs) (36, 37) and increasing oxidative stress, which can damage gut epithelial cells and reduce microbial diversity (38, 39).

Notably, we did not observe any significant differences in cardiometabolically healthy individuals between smokers and non-smokers, which contrasts a prior study indicating a significant reduction in both observed and Shannon diversity metrics (Fig. 1A) (40). This could be indicative of confounding variables such as age, diet or medication use, which can contribute to the gut microbiome differences (41, 42). In contrast, based on the observed and Chao1 alpha diversity metrics, we demonstrated that cardiometabolically abnormal individuals who smoke had significant reductions in their microbiome richness, particularly in rare species (Fig. 1B). However, this significant reduction in alpha diversity was not observed when we considered abundance using the Shannon diversity metric (Fig. 1B). This suggests that although cardiometabolically abnormal smokers have a lower absolute number of ASVs, the proportion of ASVs present in smokers and non-smokers remains similar. Therefore, ASVs lost in smokers were likely rare ASVs, and did not have a great impact on the overall proportions of ASVs composing the gut microbiome.

Furthermore, analysis of the core microbiome revealed that smokers with abnormal cardiometabolic statuses exhibit a marked decrease in the number of core microbiome members. Specifically, among the 8 ASVs uniquely absent from smoking individuals with abnormal cardiometabolic statuses, 7 belonged to the *Firmicutes* phylum (Fig. 2B). *Firmicutes* are known for their ability to degrade complex carbohydrates that are not digestible by humans via the expression of glycoside hydrolase enzymes (43, 44). This microbial metabolic activity makes more nutrients bioavailable, supplementing human digestive processes (43, 44). Additionally, *Firmicutes* produce SCFAs, such as acetate, butyrate, and propionate, which are important metabolites that provide energy and modulate the immune system (45, 46).

Among the *Firmicutes* absent from the cardiometabolically abnormal smokers, were the genera: *Streptococcus,* UCG-002*, Christensenellaceae* R-7 group, *Clostridium sensu stricto 1,* and *Blautia.* Reductions in *Streptococcus* are associated with celiac disease (47). Additionally, probiotic use has been shown to increase the abundance of *Streptococcus* and ameliorate the effects of inflammatory bowel syndrome (48). UCG-002, has been observed to be significantly reduced in individuals with chronic heart failure, suggesting a similarity of host health between cardiometabolically abnormal smokers and individuals with heart failure (49). Regarding the *Christensenellaceae R-7 group,* a study demonstrated that a traditional herbal formula fed to mice with high-fat diet-induced obesity led to anti-obesity activity, which was associated with an increase in the ASV's abundance (50). Increased levels of *Clostridium sensu stricto 1* have been identified in a murine study as being correlated with increased SCFA levels (51), suggesting potential anti-inflammatory effects. Additionally, the class of *Clostridium* has been described as an inducer of regulatory T cell (Treg) differentiation, which is associated with gut immune tolerance and anti-inflammatory effects (52). Finally, reductions in *Blautia* have been associated with chronic liver disease and hepatocellular carcinoma (53), suggesting a beneficial role. However, *Blautia* accumulation has also been correlated with both breast cancer (54) and multiple sclerosis (55), so the exact effects of *Blautia* on host health may require further characterization. Aside from *Firmicutes*, *Bifidobacterium* which belong to the *Actinobacteria* phylum, were also absent from cardiometabolically abnormal smokers. *Bifidobacteria* have recently gained attention due to their role in regulating gut homeostasis (56), and have demonstrated efficacy as a probiotic (56, 57). *Bifidobacterium* is associated with the production of acetate, which contributes to gut barrier maintenance and pathogen exclusion (58). Overall, cardiometabolically abnormal individuals who smoke have a greater susceptibility to changes in their core commensal microbiome compared to non-smokers. To determine whether a similar trend is observed for the relative expression of bacterial species in abnormal smokers relative to non-smokers, we performed differential abundance analysis.

Our differential expression sequence analysis suggests that smoking is linked to a general decrease in the relative abundance of ASVs across individuals of both cardiometabolic statuses. Interestingly, although we did not observe significant changes in terms of abundance between cardiometabolically healthy smokers and nonsmokers, smoking appears to reduce the differential relative abundance of numerous ASVs in these individuals. The majority of the ASVs experiencing this decrease belonged to the *Firmicutes* phylum, which corroborated findings from a prior cross-sectional study (59). In individuals with abnormal cardiometabolic statuses, a greater number of ASVs displayed decreases in their differential abundances relative to those who are cardiometabolically healthy. This further supports our alpha diversity and core microbiome findings, which demonstrate an increased susceptibility of cardiometabolically abnormal individuals to smoking-induced gut microbiome changes.

From the significantly differentially abundant ASVs between smokers and non-smokers that are unique to each cardiometabolic group, cardiometabolically abnormal individuals possessed ASVs associated with pro-inflammatory properties (Table S1). For instance, both *Desulfovibrio piger* and *Bacteroides ovatus* are prevalent in patients with inflammatory bowel diseases (IBDs) such as Crohn's disease and Ulcerative colitis (60, 61). *Desulfovibrio piger* is a sulfate reducing bacteria which produces hydrogen sulfate as an end product of fermentation that is toxic, mutagenic, and carcinogenic (62). *Bacteroides ovatus* has been shown to induce serum antibody responses in IBD patients, demonstrating their potential immunogenicity. Furthermore, cardiometabolically abnormal individuals contained *Clostridium perfringens*, which are commonly associated with food poisoning (63). *C. perfringens* can generate toxins, which can result in tissue damage in the small intestine, inhibit neutrophil migration and maturation, and induce neurological damage (64). Tissue damage induced by potentially harmful microbes could also contribute to the dissemination of opportunistic pathogens present in cardiometabolically abnormal individuals such as *Actinomyces* (Table S1) (65). *Actinomyces* are non-motile, filamentous bacteria which cause actinomycosis in immunodeficient individuals through the degradation of organic compounds in the small intestine (65). The presence of these potentially harmful ASVs in cardiometabolically abnormal individuals offers a potential explanation for the enhanced susceptibility of their microbiomes to smoking-induced alterations.

In cardiometabolically healthy individuals, significantly differentially abundant ASVs between smokers and non-smokers contained potentially beneficial ASVs with anti-inflammatory properties, such as *Bacteroides thetaiotaomicron* and *Bifidobacterium bifidum*. *Bacteroides thetaiotaomicron* is capable of bolstering the mucosal barrier function of the small intestine, preventing pathogen invasion (Table S1) (66). *Bifidobacterium bifidum* is capable of suppressing gut inflammation by inhibiting pro-inflammatory cytokines, and has been used for probiotic supplementation (67, 68). Furthermore, at the genus level, the gut microbiome of cardiometabolically healthy individuals contained bacteria such as *Butyricicoccus* and *Eubacterium hallii*. *Butyricicoccus* bacteria produce butyrate, a SCFA that mediates anti-inflammatory processes (69). Similarly, *Eubacterium hallii* are important for maintaining a healthy gut microenvironment because of their ability to utilize glucose and fermentation intermediates such as acetate and lactate for the production of butyrate (70). These findings indicate microbial mechanisms which could be protective for the microbiomes of cardiometabolically healthy individuals from smoking-induced changes.

Limitations We are aware of the presence of limitations in our study, which could possibly impact our findings and interpretations. The beta-diversity analysis using the unweighted UniFrac metric showed ASVs clustering into two separate PCoA plot columns (Fig. S1). This could be indicative of a confounding variable such as age, sex, BMI, or geographical location, leading to distinct clustering based on richness and phylogenetic relatedness. Moreover, there are numerous additional metadata categories associated with the dataset, whose effects have not been characterized, including fiber intake, hemoglobin level, insulin resistance, and cholesterol profile (2). Notably, stool consistency, which is characterized in the metadata, has been demonstrated to be influenced by microbial composition and richness (71). Another limitation is that smoking status was not well characterized in terms of history and tobacco consumption within the original study (2). Individual variations in smoking habits may impact the strength of our findings, especially if differences exist between cardiometabolically healthy and abnormal individuals. Furthermore, although the dataset excluded underweight individuals, those with neurodegenerative diseases, cancer, gastrointestinal diseases, or recent antibiotic use (2), other host factors can strongly influence the gut microbiome, such as alcohol consumption, bowel movement frequency, and exercise levels, which were not characterized in these study participants (42). As the original study focused on Colombians experiencing Westernization, our results may not be generalizable due to genetic and lifestyle variations between global regions. The study included adults from 18-62 years of age, with the majority being between 20-55 (2). This may further restrict the translatability of our results, as cardiometabolic abnormalities are strongly associated with older age (72). Moreover, the cross-sectional study design does not allow for the characterization of changes occuring in the microbiome throughout the ongoing Westernization process, limiting our ability to establish causality. Finally, it is important to note that the sample sizes of the subsetted categories are another limitation. Although there were similar sample sizes for the cardiometabolically healthy and abnormal groups (2), the smokers for each group were present at lower sample sizes than non-smokers, which can introduce sampling error due to unbalanced sample sizes. These limitations must be taken into consideration when interpreting the results and planning future research.

Conclusions Our findings contribute to the understanding of the effect of smoking on the microbiome of individuals with healthy and abnormal cardiometabolic statuses in the context of Westernization. We identified a consistent pattern of increased microbiome susceptibility to smoking in individuals with abnormal cardiometabolic statuses. The alpha diversity results demonstrated a significant decrease in species richness, and the core microbiome exhibited a distinct reduction in the number of taxa. Smoking was associated with a reduction in the relative abundance of a greater number of ASVs, primarily within the *Firmicutes* phylum. As discussed above, this reduction in SCFA-producing bacteria can be detrimental to host gut health. Furthermore, individuals with healthy cardiometabolic statuses possessed microbes associated with anti-inflammatory effects, known to improve host health. Overall, these findings suggest that smoking has a greater impact on the microbiome of individuals with

abnormal cardiometabolic statuses and that their microbiomes may be more susceptible to smoking-induced changes.

Future Directions To build upon our study's findings regarding the association between cardiometabolic status, smoking practices, and the gut microbiome, several short and long-term future directions should be considered.

In the short term, the gut microbiome should be analyzed using more stringent criteria for variables such as age, sex, and geographical location to provide a more accurate picture of how these factors influence the gut microbiome. Additionally, conducting a PICRUSt2 functional analysis could identify relevant metabolic pathways and functions. Specifically, due to the significant reductions we observed in the *Firmicutes* phylum in cardiometabolically abnormal smokers, exploration of the SCFA-metabolic pathways for acetate, propionate, and butyrate would provide additional insight into commensal microbial activity.

On a longer timeline, studies focused on individuals afflicted by non-communicable diseases, or those with current or recent antibiotic use should be performed, as this would provide insight into the relationships between smoking, cardiometabolic status, and the microbiome in clinically relevant contexts. Additionally, it is critical to identify the specific cardiometabolic mechanisms influenced by the bacteria identified in our study. Illustrating the current ambiguity, reduced levels of *Firmicutes* are associated with negative health outcomes, while heightened levels of *Firmicutes* are associated with obesity, so further analysis is essential to clarify their context-specific effects (73). To establish causal relationships between cardiometabolic status, smoking, and the gut microbiome, a longitudinal study design is required, as the gut microbiome is dynamic. A longitudinal study would better capture lasting changes in the microbiome and their associations with human health, while representing trends, such as Westernization, more effectively. Finally, conducting studies using populations from different countries undergoing Westernization would help determine whether our findings can be applied across regional contexts.

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CONTRIBUTIONS

Each team member contributed an equivalent amount of effort and time towards the data processing and analysis of this project. D.P. wrote the introduction and provided the core microbiome analysis. M.T. wrote the abstract and future directions and provided the alpha diversity analysis. J.W. wrote the methods section and contributed to the discussion. A.N wrote the limitations section and provided the differential abundance analysis. All team members played a part in reviewing and editing the manuscript.

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