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Smoking in the Context of Westernized Diets is Associated with a More Volatile Microbiome and Predicted Gut Function

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SUMMARY With the growing consumption of a high-fat Westernized diet in Latin America, a similarly Westernized microbiome is becoming predominant. Such a microbiome brings associations of dysbiosis and harmful metabolic products, as well as the risk of cardiometabolic disease – symptoms also associated with cigarette smokers. It is currently unclear whether the combination of a Westernized diet and cigarette smoking results in a compounded effect on the gut microbiome. In this study, we aim to investigate the synergistic effect of smoking with high blood levels of low-density lipoprotein (LDL) on the gut microbiota composition and function. Our analysis was performed to explore this potential interaction between two causes of health concern and their implications for microbiome-influenced health. Using data from a 2018 microbiome study by de la Cuesta-Zulugala et al., our analyses show no significant changes in alpha diversity metrics between smoking & non-smoking individuals and their blood LDL levels. However, at both a taxonomic and functional level smokers' microbiota appear significantly more affected by blood LDL than those of non-smokers, which were comparatively stable. Changes of note include significant increases in hallmark taxa of Westernization (*Bacteroides* and *Clostridia*), as well as downregulation of pathways relating to degradation of both aromatic compounds and D-glucarate. We postulate that the former may be in response to polycyclic aromatic hydrocarbons found in cigarette smoke, while the latter is implicated in regulation of blood cholesterol levels, suggesting a more complex interplay between smoking and measured blood LDL. Together, our findings suggest that smokers experience a more volatile gut microbiome that can be mediated through informed dietary choices.

INTRODUCTION

The growing consumption of the "Westernized" diet – one high in saturated fats, refined sugars, animal proteins, and low in fresh produce (1)– in Latin America suggests a similarly Westernized gut microbiome is becoming predominant (2). Given the role that the gut microbiome plays across human health (3–5), this Westernization warrants concern as such a microbiome is associated with dysbiosis and harmful metabolic products (6).

One marker of this dietary shift is increased blood low-density lipoprotein (LDL) levels. While low blood LDL is indicative of a healthy diet, a Westernized diet is characterized by overconsumption of saturated fats and thus elevated blood LDL, increasing risk of plaque buildup along blood vessels and cardiovascular disease (7, 8). The gut microbiota appears to regulate LDL levels through their influence on lipid metabolism (9), and in turn high-fat diets can influence microbial composition (10). Furthermore, these diets have been noted to induce shifts in gut microbiota composition that may contribute to metabolic inflammation (10). As a result, populations undergoing diet Westernization may face alterations in their gut microbiome placing them at risk for inflammatory and cardiovascular disease.

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However, there are other non-dietary factors that also significantly contribute to gut microbiome dysbiosis and disease, including cigarette smoking (11, 12). Although a smaller proportion of the Colombian population smokes than that of the United States of America, almost twice as many Colombian youths (aged 13-15) consume cigarettes compared to their American counterparts (10,11). Tobacco smoke is associated with pathobiont colonization and biofilm formation in the gut (13), including increased abundance of *Bacteroidetes, Clostridium, Bacteroides* and *Prevotella* and loss of Firmicutes and Proteobacteria compared to never-smokers (14, 15). These taxonomic shifts, most notably the change in the Bacteroides/Firmicutes ratio, are associated with inflammatory bowel disease and predisposition towards metabolic disease state and obesity (16–18). The next generation's health is put at risk by their consumption of harmful cigarette smoke, and by the consequences this causes to their microbiome.

Given the role in gut dysbiosis demonstrated by both diet Westernization and smoking, we hypothesize there is a chance that the two exhibit a compounding effect on the microbiome, resulting in a greater dysbiosis than of either alone.

This is of concern given the health consequences with which both lifestyle choices are associated, including cardiovascular diseases, gastrointestinal inflammation, and obesity (16–18). LDL levels are closely associated with cigarette smoking status, suggesting interplay between the two (19–21). Currently complex processes through which dietary LDL cholesterol interacts with gut microbiota and subsequently affects metabolic function among smokers remain a subject of ongoing research (10). One avenue for such studies requires large datasets capturing both the lifestyle and gut microbiome diversity of human populations, allowing comparison of microbiome composition and function across groups.

To investigate the extent to which diet Westernization influences the gut microbiome, de la Cuesta-Zuluaga et al. took stool samples from 411 Colombian adults and characterized their microbiota (22). The Colombian diet was noted to be shifting away from traditional (rich in complex carbohydrates such as rice, potato and corn) to Westernized, processed foods. The authors identified Western-associated marker taxa such as *Bacteroides*, *Bifidobacterium* and *Barnesiella* alongside traditional-associated *Prevotella* and *Treponema,* suggesting that population's microbiota – in reflection of their diet – was undergoing a shift from a traditional to Westernized state. The authors performed their analyses with a focus on the subsequent impact on cardiometabolic disease and obesity metrics, finding that the population's semi-Westernized microbiota suggested increased risk of both.

Using the data produced by de la Cuesta-Zuluaga et al., we sought to elucidate whether smoking and high blood LDL combined exert a synergistic effect on gut microbiota composition and function. In this study we aim to identify the extent to which cigarette smoking affects Westernization-induced alterations of the gut microbiome, using blood low-density lipoprotein (LDL) levels as a marker for the extent of Westernization. Our data suggest that smokers experience heightened LDL-associated alterations in the gut microbiome in contrast to non-smokers, underscoring the necessity for smokers to exercise caution regarding changes in an increasingly Westernized diet.

METHODS AND MATERIALS

Dataset and metadata. The dataset from de la Cuesta-Zuluaga et al. was generated by amplification and Illumina MiSeq sequencing of the 16S rRNA gene V4 hypervariable region of stool samples from 411 Colombian adults to characterize their gut microbiota (22). The study population included men and women ages 18-62 and excluded participants that took antibiotics or antiparasitics 3 months prior to enrolment, were underweight, pregnant, or diagnosed with neurodegenerative diseases, recent cancers, or gastrointestinal diseases. The study metadata included smoker status and blood serum LDL for each participant. The de la Cuesta-Zuluaga et al. study dataset is available at the SRA-NCBI under BioProject PRJNA417579. Scripts for further processing are available at Github: https://github.com/iporter-16/micb475-project2. and an overview of all processing and analyses is included below (Figure 1).

Categorization of participants. For our investigation, we binned the LDL metadata category to describe each individual's blood LDL level as "high" (>100 mg/dL) or "low" (≤100

mg/dL). The 100 mg/dL threshold was determined based on the medical standard for "healthy" blood LDL level in adults of <100 mg/dL (16). We subsequently binned the population into 4 categories ('Smoking, high LDL', 'Smoking, low LDL', 'Non-smoking, high LDL', 'Non-smoking, low LDL').

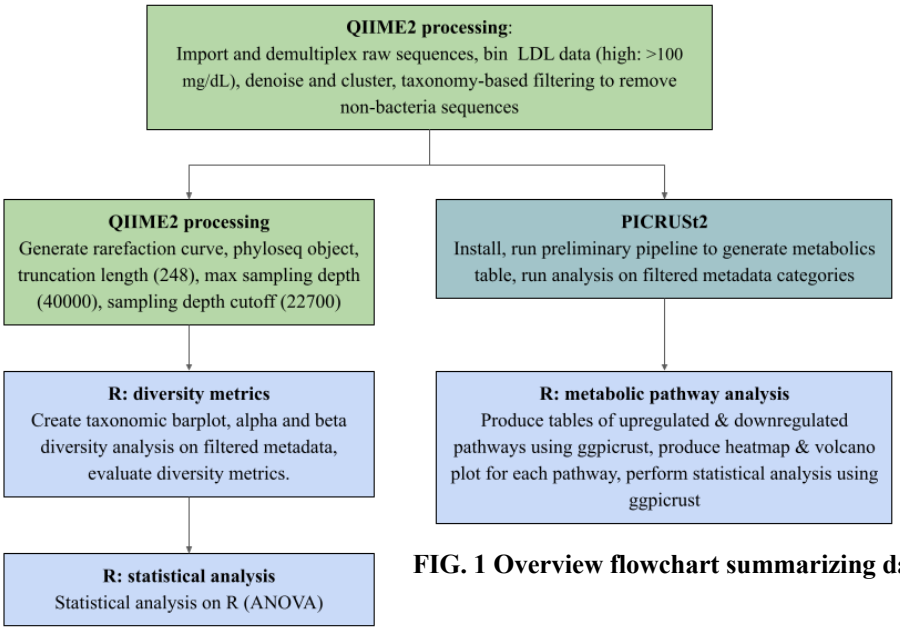

FIG. 1 Overview flowchart summarizing data analysis

Data processing using QIIME2 pipeline. We processed the 16S rRNA amplicon sequence data produced by de la Cuesta-Zuluaga et al. in Quantitative Insights Into Microbial Ecology 2 (QIIME2, v2023.7.0) (23) and demultiplexed it using the q2-demux plugin. We performed denoising and clustering using the Divisive Amplicon Denoising Algorithm 2 (DADA2) (24) with the q2-dada plugin, with a truncation length of 248 selected following sequence quality analysis (Supp Figure S1A). We trained a feature classifier using the Silva 138 reference database, the F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (′-GGACTACHVGGGTWTCTAAT-3′) primers, and truncation length of 248 (25, 26). We used this classifier to assign each sequence a taxonomic classification in QIIME2, and generated a rooted taxonomic tree using Multiple Alignment Fast Fourier Transform (27) via the q2-align-to-tree-mafft-fasttree plugin. The resulting amplicon sequencing variants (ASVs) were filtered to remove mitochondrial and chloroplast sequences. We generated an alpha rarefaction curve at a max sampling depth of 40000 and a sampling depth cutoff of 22700, chosen to preserve 8,489,800 (54.66%) features in 374 (84.81%) samples for downstream analysis (Supp Figure S1B). The rooted tree, along with ASV feature table and taxonomy table, were exported to R for subsequent analyses.

Alpha & beta diversity analysis. We created a phyloseq object using the rooted taxonomic tree, ASV feature table, taxonomy table, and binned metadata table using the phyloseq (v1.44.0) (28) and ape (v5.7-1) (29) packages. We performed alpha and beta diversity metrics on the phyloseq object using the phyloseq package, and visualized them using the following packages: tidyverse (v2.0.0), picante (v1.8.2), ggplot2 (v3.4.4), and vegan (v2.6-4) (30–33). We used one-way ANOVA tests to compare the overall Observed and Shannon's Diversity metrics between the 4 categories, and visualized themas a bar plot. For beta diversity analysis, we used permutational ANOVA (PERMANOVA) tests with the following distance matrices: Weighted UniFrac, Unweighted UniFrac, Bray-Curtis, and Jaccard. We then visualized the beta diversity as a PCoA plot using the Weighted UniFrac distance matrix.

Differential relative microbial abundance analysis. We analyzed differential relative microbial abundance in R, utilizing the generated phyloseq object (28). We incorporated a pseudocount of one into the counts before performing the differential relative microbial abundance analysis with DESeq2 (34). This addition serves to prevent undefined values and enhances the stability of variance estimation in the analysis. We assessed the statistical

significance of microbial taxa using the Wald test to compute adjusted p-values. We determined differentially abundant taxa based on a significance threshold set at an adjusted p-value of 0.01 and an effect threshold of an absolute log2 fold change greater than 2, employing the DESeq2 package (34). We visualized results in bar and volcano plots using ggplot2 (32).

Metabolic pathway analysis using PICRUSt2. We installed phylogenetic Investigation of Communities by Reconstruction of Unobserved States Version 2.0 (PICRUSt2) (35) from source and ran it to generate the PICRUSt2 functional abundance output tables for pathway classification based on the MetaCyc database (36). For analyses in R, we imported the pathway abundance table and metadata, and filtered the data to isolate smokers and non-smokers. We then performed metabolic pathway analysis was performed using the following packages: ggpicrust2 (v1.7.2), tidyverse (v2.0.0), ggprism (v1.0.4), patchwork (v1.1.3), DESeq2 (v1.42.0), and ggh4x (v0.2.6) (30, 34, 37–40). We performed pathway differential abundance analysis (DAA) on the LDL category group using the DESeq2 method for smokers and non-smokers, and annotated MetaCyC pathway results in each case. We visualized significant ($p < 0.05$) pathways from the metabolic pathway abundance results using pathway PCA plots and log2 fold change bar plots.

RESULTS

No significant changes in diversity were observed between dietary and smoking categories. To assess the overall impact of smoking and blood LDL level on gut microbiome diversity, we measured alpha and beta diversity for the four population categories ("Non-smoking, high LDL": n = 31, "Non-smoking, low LDL": n = 295, "Smoking, high LDL": n = 37, and "Smoking, low LDL": n = 9). For alpha diversity measurements, both Observed features (Figure 2A) and Shannon's Diversity Index (Figure 2B) showed no significant differences (One-Way ANOVA, $p<0.05$ cutoff) between the four categories suggesting no significant differences in community richness and abundance. Similarly, the beta diversity analyses measured using the Weighted UniFrac distance matrix and compared using PERMANOVA showed no significant difference between the groups. Upon PCoA analysis, samples did not display distinct clustering according to LDL category (Figure 3) further demonstrating that there was no significant difference in microbial composition.

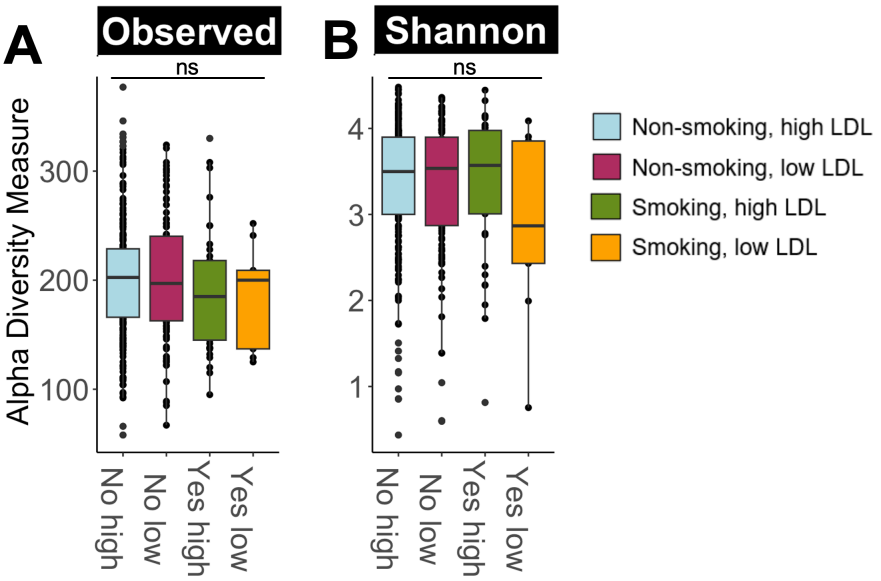

FIG. 2 No significant effect on alpha diversity observed between different smoking and LDL populations. Alpha diversity measured using Observed Features **(A)** and Shannon's Diversity Metric **(B)** shows no significant difference (ns) between four population categories, assessed using One-Way ANOVA with minimum $p<0.05$ cutoff.

Distinct LDL-associated alterations in gut bacterial composition evident in smokers compared to non-smokers. Despite no difference found in diversity metrics, changes in taxonomic abundances are also indicative of gut microbiome alteration. As a result, we performed DESeq2 analysis to identify significantly altered taxa. We found17 ASVs were upregulated and 13 downregulated in smokers with high LDL relative to the smokers with

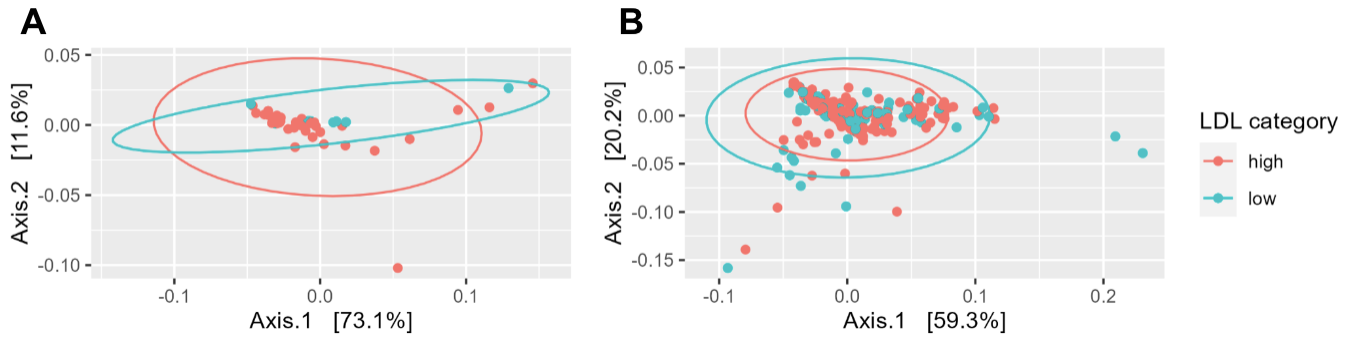

FIG. 3 No significant effect on beta diversity observed between different smoking and LDL populations. PCoA plots of Weighted UniFrac distance to visualize beta diversity between smoking and LDL groups show no distinct clustering difference between high and low LDL blood levels of **(A)** smokers or **(B)** non-smokers.

low LDL (Figure 4A). The most downregulated taxa included *Prevotella, Muribaculaceae*, and *Lachnospiraceae*, while the most upregulated included *Howardella*, *Bacteroides*, and *Clostridia* (Figure 4B). Conversely, in the non-smoking high LDL group only 2 ASVs within one genus (RF39) displayed significant changes as both upregulated and downregulated taxa compared to the non-smoking low LDL group (Supp Figure S3B). These findings suggest that smokers exhibit larger LDL-induced alterations in the gut microbiome composition than non-smokers.

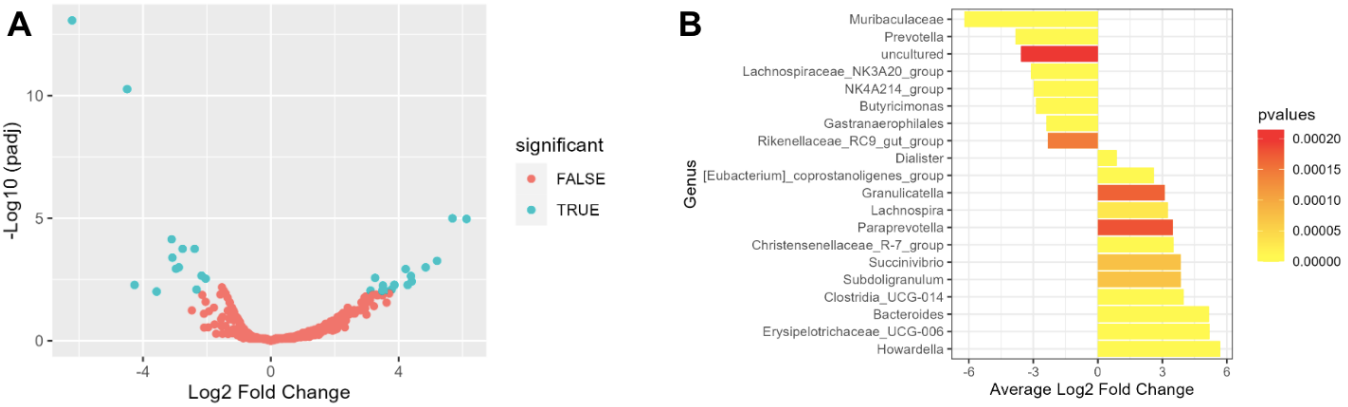

FIG. 4 Smokers experience exacerbated LDL-associated up and down regulation of gut bacteria relative to smokers with low LDL. (A) Comparing differential abundances of bacterial ASVs labelled at the genus level. Volcano plot blue dots: |Log2 fold change|> 2 of smokers with high LDL vs. low LDL, p adj < 0.01. **(B)** Taxa bar plot. Differential expression of genera and corresponding log2 fold changes in smokers with high LDL compared to smokers with low LDL. p adj < 0.01 and |Log2 fold change| > 2.

Smokers experience exacerbated LDL-associated alterations in gut metabolic pathways compared to non-smokers. As taxonomic changes may be associated with functional changes to the gut microbiota, we next investigated the changes in predicted metabolic pathways between smokers and non-smokers with varying blood LDL levels using PICRUSt2. Notably, we observed that smokers exhibited 13 upregulated pathways (log2 fold change >1) upon adopting a high blood LDL level (Figure 5). This is contrasted with only one pathway upregulated in non-smokers with high blood LDL levels. The upregulated pathways taken from smokers include the degradation of aromatic compounds, and of D-glucarate and D-galactarate, while the singular pathway upregulated in the non-smokers cohort is the 3-phenylpropanoate and 3-(3-hydroxyphenyl) propanoate degradation pathway. These trends suggest smokers have a more volatile microbiome pertaining to gut function compared to that of non-smokers.

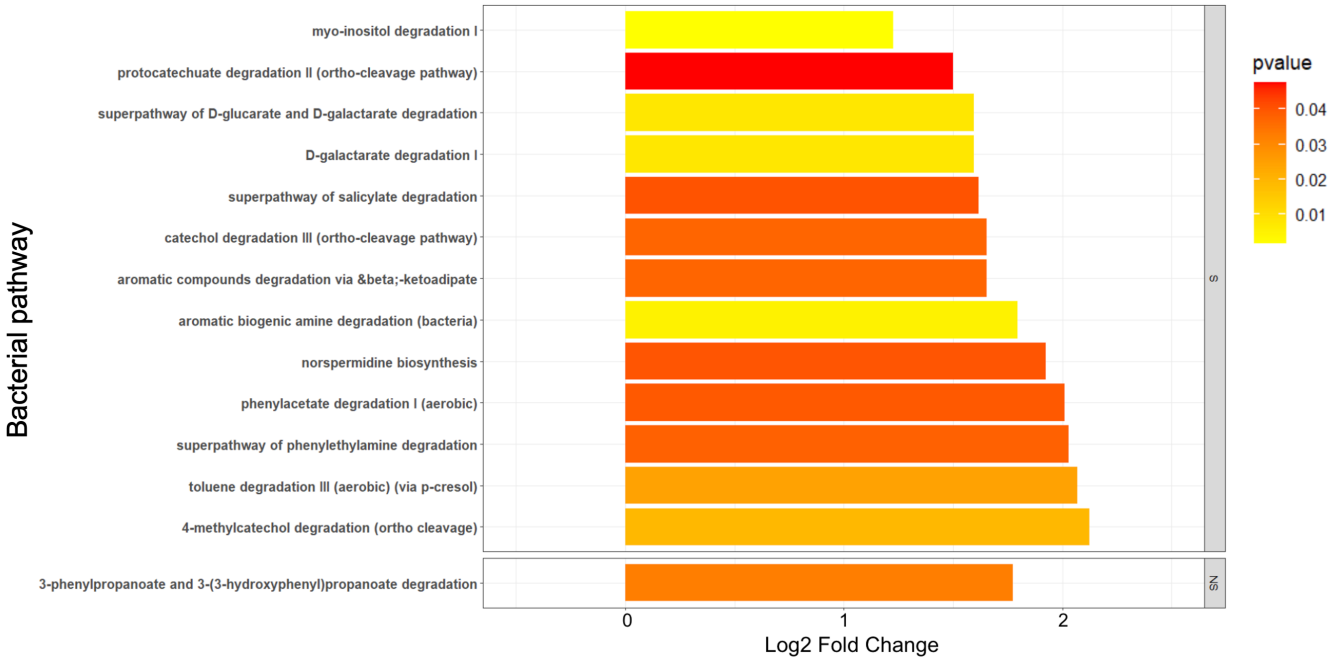

FIG. 5 Smokers experience exacerbated LDL-associated alterations in gut metabolic pathways compared to non-smokers. Pathway differential abundance analysis via DESeq2 method. PICRUSt2-generated bar plot of high LDL-associated metabolic pathway changes respective to the low LDL healthy reference. S = Smoking cohort, NS = Non-smoking cohort. Filtered by |log2 Fold change| > 1; significance assessed with Wald test using $p < 0.05$.

DISCUSSION

In this study, we aimed to unravel the complex interplay between smoking and dietary changes on the gut microbiome in a Colombian population undergoing Westernization. While prior literature has extensively characterized the isolated effects of smoking on gut microbial diversity (11, 12), the interactions between smoking and LDL levels amid the broader trend of Westernization remain poorly understood. Specifically, we investigated the associations between smoking status and blood LDL levels to find their implications in healthy gut microbial composition and metabolism.

To investigate the gut microbial changes due to smoking status and blood LDL levels, we examined the alpha and beta diversity metrics between groups. Respectively, changes in community richness and dissimilarity between communities can be explained using these metrics. The LDL blood levels and smoking conditions do not demonstrate a significant effect on the alpha and beta diversity of the gut microbiome (Figure 2, 3). Some studies have shown similar observations in the non-significant effect on alpha diversity (41). However, there are also past studies showing that smokers have a significant change in alpha diversity (42) and a significant difference in UniFrac-based beta diversity compared to never-smokers (14), which is inconsistent with our observations. The inconsistency might stem from a lack of control over the cities where the Colombian population resides, considering that cities and their associated diet habits and environments exert a substantial additional influence on microbiome diversity (43, 44). The alpha diversity analyses showed large variation within categories, and the PCoA analyses included many outlying points, which supports the concern of extraneous and confounding variables affecting our conclusions. As a result, the diversity analyses could benefit from greater filtering and accounting for differences in metadata prior to concluding significance. Although the diversity metrics do not show a significant difference between LDL and smoking conditions, there is a different set of upregulation and downregulation in gut microbiome genera patterns between smokers and non-smokers (Figure 4B, Supp Figure S3B).

Of the bacterial genera that significantly differed in smokers with high LDL, the genera *Bacteroides*, *Clostridia*, and *Prevotella* (Figure 4B) are strong markers of diet and lifestyle (45). We found that *Bacteroides* and *Clostridia* were upregulated in smokers with high LDL

which is consistent with previous studies that describe these genera as more common in Westernized diets (10, 46). The significant upregulation of *Bacteroides* and *Clostridia* may be attributed to the genus' ability to tolerate bile acids which is common in the gut of increased meat intake seen in the Western diet (46) – repeating these studies with a focus on protein intake could be a promising next step. The metabolism and homeostasis of bile acids and cholesterol is integral to the prevention of the pathogenesis of cardiometabolic diseases such as atherosclerotic cardiovascular disease (47). With the upregulation of these bacteria in the gut, it is important to assess their health impacts overall.

Bacteroides are typically commensal bacteria, but their overgrowth due to dietary habits can result in increased degradation of mucus, contributing to a pro-inflammatory state at the body site (48). *Bacteroides* can outcompete other bacteria and employ the most antibiotic resistance mechanisms, harboring concerns regarding their overgrowth and dysregulation (49). Additionally, biofilms produced by certain *Bacteroides* species may lead to cancer formation with their influence on increasing concentrations of reactive oxygen species (48). Our findings suggest that smokers have an exacerbated LDL-associated upregulation of *Bacteroides* (Figure 4A-B), necessitating the need for smokers to monitor their LDL intake more keenly.

Our findings indicate that *Clostridia* bacteria were also upregulated similarly to *Bacteroides* within smoking, high blood LDL individuals (Figure 4A-B). In times of gut dysregulation, overgrowth of species like *Clostridioides difficile* may lead to severe infection, entailing functional gastrointestinal disorders (46). Species of *Clostridia* in the gut also contribute to human diseases such as gas gangrene, tetanus, botulism, pseudomembranous colitis, and food poisoning (50). This should further incentivize smokers with high LDL levels to be aware of the LDL content in their diet.

On the other hand, the genus *Prevotella* was downregulated in the same population. According to the literature, *Prevotella* bacteria are significantly increased in tobacco smokers and former smokers (12, 51). In the context of diet Westernization, represented by our categorization of blood LDL levels, the genus tends to dominate a non-Westernized gut microbiome, but is decreased in Westernized populations which is in line with our findings (51). Further research finds that fat intake negatively correlates with *Prevotella* possibly because of its sensitivity to bile acids, which are upregulated in individuals who partake in high-fat diets (52). From this, we speculate that dietary fat intake has a greater influence on the regulation of *Prevotella* in the gut compared to smoking status. The *Prevotella* genus has been shown to be anti-inflammatory and protective against other bacteria by competing for fiber (46). However, various studies have also shown conflicting results on the role of *Prevotella* and dietary patterns on gut inflammation (53) and cardiometabolic health, including regulation of blood cholesterol and colitis susceptibility (51, 54). These conflicting results can be explained by inconsistencies in taxonomic designations and species boundaries, as well as strain-specific metabolic diversity (53). Again, we find that a high blood LDL level in combination with smoking shows markedly greater downregulation of *Prevotella* (Figure 4A-B). Whether the downregulation of *Prevotella* in the gut microbiome is beneficial or not is unclear and further studies are needed to understand the conflicting results in previous studies and the current study.

In terms of functional changes, most significantly upregulated pathways in smokers (with high LDL) are related to degradation of aromatic compounds (Figure 5). Regrettably, the literature has no extensive research linking the degradation of aromatic compounds in the gut to human health. However, we speculate that the upregulation of these pathways is directly implicated from smoking. For smokers, tobacco products contain polycyclic aromatic hydrocarbons (PAHs) that can be released during burning, and subsequently swallowed, which can then be a target for degradation (55). These compounds are known to be mutagenic, carcinogenic, and teratogenic (56). PAHs – serving as a distinct indicator of smoking status – along with elevated blood LDL levels, play a role in the mentioned pathway alterations. It may signify an upturn in the abundance of bacteria associated with the degradation of aromatic compounds. Indeed, this is expected of genera including *Howardella* (57), *Clostridia* (57), and *Bacteroides* (58) which we find significantly upregulated in the DESeq2 analyses. Both *Clostridia* and *Bacteroides* are known to degrade PAHs (52, 53). PAHs are known to be lipid-soluble, to which we speculate that a Westernized diet may increase

gastrointestinal absorption (59). Additionally, it has been shown in murine models that increased exposure to benzo[a]pyrene, a PAH, induces compositional changes in gut diversity and abundances, and leads to increased penetrability and inflammation in the ileal segment mucosa (60). In contrast, the only pathway that exhibited a significant upregulation in the non-smoking, high LDL population was 3-phenylpropionate and 3-(3-hydroxyphenyl) propionate degradation (Figure 5). While these pathways are also categorized into the aromatic compound degradation cluster, the degradation of these compounds ultimately yields valuable nutrients like succinate, acetyl-CoA, and pyruvate for the gut lumen to absorb (61).

Additionally, our functional analysis shows smokers with high blood LDL experience significant upregulation of D-glucarate and D-galactarate degradation pathways relative to those with low blood LDL (Figure 5). These pathways are associated with similarly upregulated genera including *Clostridia* and *Bacteroides* (62, 63). Interestingly, they have also been associated with *Prevotella* (61), which we found to be downregulated in the high LDL group. Cholesterol degradation is associated with D-glucarate (64), making it possible that the elevated blood LDL levels in these individuals may be a result of their altered microbiomes. This suggests that smoking fosters a microbiome inhibitory to the maintenance of healthy cholesterol levels, adding a layer of complexity to the interplay between the two variables.

Limitations We acknowledge the existence of limitations in our study, which may influence our findings and interpretations. One of the major limitations is the unequal sample size between the categories of interest. For example, there are significantly more non-smokers (n=326) compared to smokers (n=46). Within the smoking population, only 9 individuals belong to the low LDL category, and 37 belong to the high LDL category, which may reduce the statistical power of the analysis. This makes it challenging to draw definitive conclusions and introduces type II error, in which the null hypothesis is not rejected when it is actually false. This limitation also constrained the exploration of other dietary categories.

Another limitation of this study is that our computational analysis relied on a single dataset collected by de la Cuesta-Zuluaga et al., which comprised individuals residing in several of Colombia's major urban cities. As the genetic composition of the population – along with environmental conditions – can influence the microbiome diversity, these factors may be Colombia-specific (65, 66). Hence, trends observed in this study may not represent the global microbiome diversity.

Finally, the presence of confounding variables within our dataset represents a significant limitation. The smoking status and blood LDL are the only factors explored in our study to study the changes in microbiome diversity, but other variables affecting the population – such as age and sex – could act as confounders (4, 67). In particular, it has been shown that the city lived in has a significant effect on the microbiome composition within this population (43), which we did not account for in our analyses. The unequal distribution of these variables among our study groups may lead to biased associations, making it challenging to isolate the specific impacts of smoking and LDL status on the gut microbiome.

Conclusions Our research findings provide insights into how a Westernized diet and smoking habits influence overall gut microbiome diversity. Although alpha diversity analysis didn't show significant changes in species richness and abundance, and beta diversity analysis indicated no major differences in microbial composition across diversity levels, we observed specific alterations in upregulated pathways and differentially regulated bacterial species. Our investigation highlighted that smokers display an increased presence of *Clostridia* and *Bacteroides*, accompanied by an upregulation in the degradation of D-glucarate. The association between *Bacteroides* and D-glucarate breakdown, linked to cholesterol metabolism, suggests a potential reduction in cholesterol breakdown among smokers, potentially leading to higher LDL levels in the bloodstream. Additionally, smokers exhibited a decrease in *Prevotella*, known for their anti-inflammatory properties that contribute to overall host health. In conclusion, these findings caution smokers to remain vigilant about their LDL levels due to the observed volatile nature of the smoker's gut microbiota. This

emphasizes the importance of monitoring cholesterol levels among individuals who smoke, considering the identified microbiome alterations.

Future Directions This study demonstrates that smoking and elevated blood LDL may act synergistically on the gut microbiome, resulting in exacerbated functional changes. A suitable next step would be further investigation into the participants' metabolite profiles in order to validate these changes and confirm their health implications. In particular, the level of D-glucarate and cholesterol can be sampled and investigated to confirm whether the upregulation in D-glucarate degradation genuinely influences blood LDL (64).

These analyses would benefit from an expanded dataset including – and accounting for – broader geographic reach. This expansion would help mitigate the above-mentioned limitations of our current dataset, facilitating a more equitable distribution of participants across dietary categories and allowing a more nuanced classification of dietary habits. Initially our investigation extended to examine the potential impact of fiber intake on microbiome diversity (Supp Figure S2A-B). However, in contradiction to prior studies (68) these analyses failed to demonstrate statistical significance between smoking and fiber intake, potentially as a result of small sample size. Fiber and other dietary categories such as protein intake should be revisited with such an expanded dataset, as literature suggests their impact on the gut microbiome is akin to that of smoking and may thus be exacerbated by smoking status (46, 69, 70).

In addition, our binary classification of LDL levels into high or low around a 100 mg/dL cutoff lacks a middle category, limiting the ability to capture nuances in lipid profiles. Excluding participants within a certain range around set dietary thresholds could provide a more detailed assessment. Introducing an intermediate category would furnish us with insights into the diversity changes among various LDL categories, not solely the extremes.

Finally, potential confounding variables such as age, sex, and geographical location (i.e. city) can be identified. It has already been demonstrated that participants' city of residence has a significant effect on their microbiome composition (43), as can an individual's sex hormones (71). Future directions should involve identification and controlled analysis of these confounding factors – such as stratification by sex and location – to allow a more rounded interpretation of observed relationships. Recognizing and addressing these confounding factors would allow greater interpretations of the observed relationships in the context of the broader Westernization trends in the Colombian population.

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CONTRIBUTIONS

All team members made equal and substantial contributions in terms of effort and time dedicated to the creation, data processing, and analysis for this project. IP performed functional analyses in PICRUSt2, and contributed to writing the abstract, introduction, methods, results, discussion, and references. AW performed differential abundance analysis of ASVs and DESeq2 in R and contributed to writing the methods and materials, results, discussion, and future directions. SD performed QIIME2 processing, pathway differential analysis via DESeq2, and contributed to writing the methods, results, and discussion. SA performed QIIME2 processing, beta diversity analysis, PICRUSt2 functional analyses, and contributed to writing methods, results, and discussion. TW performed assigned metadata filtering/ wrangling roles, alpha diversity and DESeq2 analysis in R, and contributed to writing methods, results, discussion, limitations, conclusion, and future directions. All team members contributed to reviewing and editing the manuscript.

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