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Gut Microbiome Mediates Age- and Sex-Divergent Effects of Environmental Exposures on Human Health

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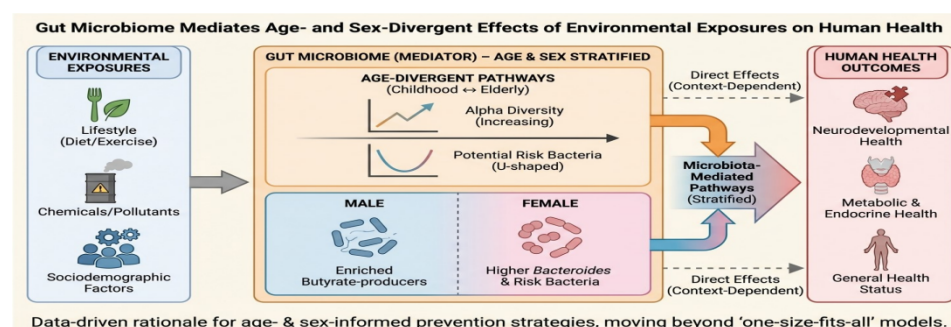
Keywords

gut microbiome;
host-microbe interactions;
mediation analysis;
sex differences;
environmental exposures

Highlights

- Age and sex stratify gut microbiome responses to environmental exposures.
- Mediation analysis reveals distinct pollution-microbiome-health pathways.
- ASD risk from Endosulfan is microbiota-mediated in females, direct in males.
- Diversity rises with age while risk bacteria exhibit a U-shaped trajectory.

Abstract: The gut microbiome acts as a critical interface between environmental exposures and human health, yet the influence of intrinsic host factors on this interaction remains poorly understood. We hypothesized that the microbiome's role as a mediator of environmental risks is fundamentally stratified by host age and sex. Integrating 16S rRNA gene sequencing from 13,991 American Gut Project participants with extensive geospatial environmental datasets, we performed stratified regression and formal mediation analyses on 86 environmental/personal factors and 24 self-reported health outcomes. We identified distinct, age- and sex-dependent microbial trajectories; alpha diversity increased with age, while the abundance of potential risk bacteria exhibited a U-shaped distribution, peaking in children and the elderly ($p < 0.001$). Females had a higher abundance of *Bacteroides* and potential risk bacteria, whereas males were enriched in butyrate-producing bacteria ($p < 0.001$). The influence of environmental factors was highly stratified; for example, the impact of diet and exercise on alpha diversity was strongest in children, while travel's impact was greatest in the elderly ($q < 0.05$). Mediation analysis identified potential stratified risk pathways. The microbiome-mediated risk of thyroid dysfunction from POPs was significant only in older adults ($p < 0.05$). Furthermore, the risk pathway for ASD linked to Endosulfan exposure was sex-dependent: a direct toxicological effect was significant in males, whereas the primary pathway in females was microbiota-mediated. These stratified pathways provide potential mechanistic insights into well-known demographic disparities in human diseases. Our findings provide a data-driven rationale for moving beyond “one-size-fits-all” models toward age- and sex-informed strategies for disease prevention and public health.



1. Introduction

Human health exhibits profound variability, where susceptibility to disease is shaped by a complex interplay between environmental exposures and individual biology. The gut microbiome has emerged as a critical, yet enigmatic, mediator in this axis, translating external factors—from diet to pollutants—into physiological responses that can either maintain health or precipitate disease [1–4]. However, the outcomes of these interactions are often inconsistent across populations. Understanding the principles that govern this variability is a central challenge in biology and medicine and is essential for developing effective strategies for disease prevention.

The dynamic nature of the gut microbiome is well-documented. Its composition follows a dramatic trajectory across the lifespan, from the rapid colonization of infancy to the diversification of adulthood and a potential loss of resilience in the elderly population [5,6]. Likewise, biological sex imprints distinct microbial signatures, an effect thought to be mediated by the influence of sex hormones like estrogen and testosterone on the gut environment and host immunity [7–9]. However, despite this knowledge, in the health-microbiome association studies, age and sex have typically been treated as independent variables or simple confounders to be statistically controlled [10,11]. This approach overlooks a more fundamental possibility: that age and sex do not merely correlate with microbial changes, but actively reshape the rules of engagement between the host, the microbiome, and the environment.

We hypothesized that age and sex are not simply variables, but are foundational biological contexts that shape the microbiome's potential role as a mediator of environmental risk. This would mean the microbiome acts as a context-dependent filter, where the same environmental input is processed differently—and channeled towards different health outcomes—depending on an individual's demographic stratum. Resolving whether such stratified pathways exist is essential for understanding the biological basis of demographic disparities in health and for moving beyond “one-size-fits-all” models of disease risk.

Here, we test this hypothesis by conducting a systematic analysis of the pathways linking 86 environmental factors, 10 microbial indices, and 24 distinct health outcomes in a global cohort of 13,991 individuals. Our analysis first reveals robust, subgroup-specific associations, showing how distinct factors like diet and air pollution differentially shape the gut microbiome in males versus females and across the lifespan. Building on these foundational links, our mediation modeling then suggests how these interactions may translate to health outcomes. These analyses provide a new framework for investigating disease etiology and a

rich, data-driven basis for understanding demographic disparities in human health.

2. Methods

2.1. Study Cohort and Data Sources

This study utilized data from the publicly available American Gut Project [12] (AGP, <http://ftp.microbio.me/AmericanGut/latest> (accessed on 15 January 2024)), a large, crowdsourced citizen science initiative. Participants in the AGP are self-selected and typically purchase a sampling kit to contribute their data for open research. While the cohort predominantly consists of individuals from North America and Europe, it represents a global initiative with significant participation from Asian and Pacific regions, providing a diverse international dataset for analysis. A detailed summary of the demographic characteristics of our final analytical cohort, including age distribution, sex ratio, and geographical location, can be found in Supplementary Table S1.

The microbial data provided by the AGP consisted of genus-level feature tables, generated by the QIITA platform using a standardized QIIME 2 pipeline [13]. This process involved sequence denoising with DADA2 to generate Amplicon Sequence Variants (ASVs), which were assigned taxonomy against the Greengenes database (version 13.8) before being collapsed to the genus level. To create our analytical cohort, we filtered for fecal samples and excluded individuals with missing age, sex, or geolocational data, resulting in a final cohort of 13,991 individuals.

2.2. Personal Characteristics and Questionnaire Data Processing

Personal characteristics, lifestyle habits, and health status were derived from the AGP questionnaire data. To facilitate quantitative analysis, all categorical and ordinal responses were converted to a numerical scale. A standardized coding scheme was applied as follows: binary responses (‘yes’/‘true’, ‘no’/‘false’) were coded as 1 and 0, respectively. For questions assessing the frequency of behaviors, such as diet and lifestyle, we transformed the ordinal categories into a continuous scale representing the average daily frequency. This was implemented by dividing the representative number of occurrences for each category by the corresponding number of days. The specific conversions were as follows: “Never” was coded as 0; “Rarely (several times a month)” as ≈ 0.066 (calculated as 2 occurrences/30.4 days); “Occasionally (1–2 times a week)” as ≈ 0.214 (1.5 occurrences/7 days); “Often (3–5 times a week)” as ≈ 0.571 (4 occurrences/7 days); and “Everyday” as 1. All other non-frequency ordinal variables were converted to a numerical scale, such as from 1 to 5, for analysis.

2.3. Environmental Exposure Assessment and Geospatial Integration

To profile the environmental exposures for each AGP participant, we developed a multi-step pipeline to link their geolocal data to public environmental databases.

First, participant location was determined from the latitude and longitude coordinates in the AGP metadata. These coordinates were programmatically converted to corresponding city-level information using a custom R script interfacing with the Google Geocoding API. We then assigned exposure values by systematically extracting data from numerous public databases covering air pollutants, soil characteristics, climate metrics, mycotoxins, demographics, and persistent organic pollutants (POPs) (Supplementary Table S1). This extraction process handled diverse data structures, including tabular data, Esri grid formats (e.g., TIF/TIFF, NetCDF), and vector-based shapefiles. To ensure consistent data extraction using ArcMap (version 10.8), shapefiles were first converted to the Esri grid format, and raster layers were created from NetCDF files to facilitate point-by-point data sampling.

For air pollutants such as PM_{2.5}, NO₂, CO, and O₃, we utilized satellite-derived data from NASA's EARTHDATA platform. For PM₁₀ and SO₂, we linked each participant's city to monitoring station data from the U.S. Environmental Protection Agency (US EPA) Air Quality System. For Persistent Organic Pollutants (POPs), we used data reported under the Stockholm Convention's Global Monitoring Plan and applied spatial interpolation models to estimate concentrations for each location.

For mycotoxin exposure, which cannot be measured directly, we developed and applied a two-step estimation model. This model estimated each participant's exposure to six major mycotoxins: aflatoxin B1 (AFB1), fumonisin (FB), zearalenone (ZEN), deoxynivalenol (DON), ochratoxin A (OTA), and T-2 toxin. The model was based on public data on mycotoxin detection rates and concentrations in animal feeds and food commodities across various countries from 2008–2017. First, a representative concentration (C) for each mycotoxin (k) in each participant's country/region (j) was calculated by multiplying the regional detection rate (D_{jk}) by the median concentration of detected mycotoxins (M_{jk}):

$$C_{jk} = D_{jk} \times M_{jk} \quad (1)$$

Second, this regional concentration was used to calculate an estimated personal exposure score (E) for each mycotoxin. This score was derived by weighting the regional concentration by the participant's self-reported intake frequency (F) for three primary food categories known to be sources of exposure: grains (weight, $W = 0.5$), dairy products ($W = 0.3$), and nuts ($W = 0.2$), reflecting the established hierarchy of these food groups as primary vectors for mycotoxin contamination. The final individual exposure score for mycotoxin k (E_k) was

calculated as the regional concentration multiplied by the sum of the weighted intake frequencies for the three food categories:

$$E_k = C_{jk} \times \sum_{i=1}^3 (F_{ij} \times W_i) \quad (2)$$

To create meaningful exposure variables from this raw data, we applied specific modeling and temporal averaging approaches. To account for exposure history and potential lag effects in the AGP cohort (sampled 2013–2018), we calculated a three-year average exposure level preceding each participant's sample collection date for variables like air pollutants and POPs. The initial data integration yielded a large set of environmental features, many of which were highly inter-correlated. To create a robust and non-redundant feature set for our models, we performed a systematic curation process. We first conducted a Spearman correlation analysis for all environmental variables using the psych package (version 2.4.1) in R (version 4.3.2). To mitigate multicollinearity, we iteratively removed variables with a correlation coefficient $|\rho| > 0.85$. This procedure resulted in a final set of 36 distinct environmental and demographic variables for analysis, including 6 mycotoxin, 8 POP, and 6 air pollutant, and 16 other climatic and geographic variables.

Finally, after integrating all personal, microbial, and curated environmental data, we defined our final analytical cohorts. We excluded samples with more than 50% missing key questionnaire information, resulting in 13,130 samples for age-based analyses and 12,794 samples for sex-based analyses. A detailed summary of the demographic characteristics for these final cohorts, along with descriptive statistics for all curated variables, can be found in Supplementary Tables S1–S3.

2.4. Data Standardization and Variable Curation

We performed a two-step filtering process to select a robust subset of predictors. First, to mitigate multicollinearity, we removed variables with a Spearman correlation coefficient > 0.85 . Second, to enrich for relevant factors, we performed univariate linear regressions against the ten microbial indices and selected 50 variables based on statistical significance and relevance to our study's hypotheses. This curated set included 8 personal characteristics, 13 lifestyle factors, 7 antibiotic/vitamin-related variables, 16 dietary frequency variables, and 6 alcohol consumption variables.

To comprehensively capture different facets of the gut microbiome, we engineered a suite of ten distinct microbial indices from the genus-level data. These indices included measures of alpha diversity (Shannon index, Observed Features, and Fisher's alpha), which were calculated using QIIME 2 (version 2024.2). We used the first two principal coordinates (PCoA1 and PCoA2) from a Bray-Curtis dissimilarity analysis as proxies for overall

community structure (beta diversity). We also calculated two community-wide taxonomic ratios: the *Firmicutes*-to-*Bacteroidetes* (F/B) ratio and the Gram-positive-to-Gram-negative ratio. To represent potential dysbiosis, we created potential risk bacteria as the summed relative abundance of three genera consistently associated with opportunistic infections: *Eggerthella*, *Anaerotruncus*, and *Klebsiella*. Finally, we calculated the summed relative abundances of two key putative functional guilds—Butyrate-producing genera and Lactate-producing genera—based on established literature. Butyrate-producing bacteria included *Butyricimonas*, *Odoribacter*, *Anaerostipes*, *Agathobacter*, *Anaerobutyricum*, *Butyrivibrio*, *Coprococcus*, *Roseburia*, *Shuttleworthia*, *Butyricicoccus*, *Faecalibacterium*, *Flavonifractor*, *Pseudoflavonifractor*, *Oscillibacter*, *Subdoligranulum*, *Subdoligranulum variabile*, and *Eubacterium ventriosum*. Lactate-producing bacteria included *Bifidobacterium*, *Lactobacillus*, *Streptococcus*, *Enterococcus*, *Leuconostoc*, *Lactococcus*, and *Pediococcus*.

2.5. Stratified Association and Mediation

Our primary goal was to identify significant environment-microbe associations within specific demographic subgroups. To achieve this, we first stratified the cohort by age and by sex. The five age-based strata were: child (1–12 years), teen (13–19), young adult (20–39), middle-aged adult (40–59), and elderly (≥60). The sex-based strata were male and female.

Within each of these distinct subgroups, we performed a two-step screening procedure using Stata (version 17.0). First, each of the 86 curated environmental and personal characteristics was tested for linear or logistic regression with each of the 10 microbial indices using univariate linear regression for that subgroup. For the raw *p*-values of multiple tests within each subgroup, the Benjamini-Hochberg (BH) method was used for FDR correction to control the false discovery rate in multiple tests. Only associations with an FDR-corrected *q*-value < 0.05 were considered significant. This subgroup-specific process allowed us to identify the key variables to carry forward into the final mediation analyses (Supplementary Figure S1).

The dependent variables for our mediation models were derived from the self-reported health histories available in the AGP dataset. We initially identified 36 variables related to participant health, including diagnoses like Small Intestinal Bacterial Overgrowth (SIBO) and metrics such as Body Mass Index (BMI). To ensure our analysis focused on distinct disease states, we performed a curation step to remove variables representing heterogeneous or poorly defined categories (e.g., ‘liver disease’, which encompasses many conditions). Following the exclusion of 12 such variables, a final set of 24 specific health conditions was retained for all subsequent mediation analyses.

To test our central hypothesis that the gut microbiome mediates the effects of environmental exposures on health outcomes, we employed a formal mediation analysis framework. This approach decomposes the total effect of an independent variable (*X*, an environmental factor) on a dependent variable (*Y*, a health outcome) into two components: a direct effect (path *c'*) and an indirect effect that operates through a mediating variable (*M*, a microbial index). The indirect effect is quantified as the product of two path coefficients: the effect of *X* on *M* (path *a*) and the effect of *M* on *Y* while controlling for *X* (path *b*). This framework is represented by a set of three core regression equations:

$$Y = cX + e_1 \quad (3)$$

$$M = aX + e_2 \quad (4)$$

$$Y = c'X + bM + e_3 \quad (5)$$

*e*₁, *e*₂, and *e*₃ are the error terms of the three equations, respectively.

All mediation analyses were conducted in R (version 4.3.2) using the mediation package. The specific regression models used for each path were chosen based on the nature of the dependent variable in that equation (i.e., continuous for microbial indices, binary for most health outcomes):

For continuous dependent variable (*Y*), we followed the standard formal mediation procedure. Significance of the indirect effect (*a* × *b*) was tested using a non-parametric bootstrapping method (1000 resamples) to generate 95% confidence intervals (CIs). A CI that does not contain zero indicates a statistically significant mediation effect.

For binary health outcomes (*Y*), We used a modeling approach appropriate for dichotomous outcomes using the medci function of the R mediation package. Path ‘*a*’ (*M* ~ *X*) was modeled using linear regression, as the microbial mediators (*M*) were continuous. The outcome model (*Y* ~ *X* + *M*, path ‘*c*’ and ‘*b*’) was modeled using logistic regression. Significance of the indirect effect was again determined using bootstrapping (1000 resamples) to generate confidence intervals.

$$Y' = i_5 + cX + \varepsilon_5 \quad (6)$$

$$M = i_6 + aX + \varepsilon_6 \quad (7)$$

$$Y'' = i_7 + c'X + bM + \varepsilon_7 \quad (8)$$

$$Y' = \text{Logit}P(Y = 1|X) = \ln \frac{P(Y = 1|X)}{P(Y = 0|X)} \quad (9)$$

$$Y'' = \text{Logit}P(Y = 1|X, M) = \ln \frac{P(Y = 1|M, X)}{P(Y = 0|M, X)} \quad (10)$$

A core component of our study was to perform all mediation analyses separately within each age and sex-based subgroup. This stratification allowed us to directly compare the magnitude and significance of the direct (c') and indirect ($a \times b$) effects across different demographic contexts. Prior to analysis, all continuous variables were standardized (mean-centered and scaled by standard deviation) to facilitate the comparison of effect sizes. The results of the mediation were categorized based on the significance and direction of the direct and indirect effects, allowing us to identify instances of full mediation, partial mediation, or inconsistent mediation (Supplementary Figure S2, Supplementary Table S4).

3. Results

3.1. Age Shapes Distinct Gut Microbial Trajectories Across the Lifespan

To establish the impact of aging on the gut microbiome, we first examined microbial community metrics across five distinct age groups, including child (1–12 years), teen (13–19), young adult (20–39), middle-aged adult (40–59), and elderly (≥ 60). We observed clear, age-dependent trajectories in both community diversity and the abundance of key functional guilds (Figures 1A and S3, Supplementary Table S5).

Alpha diversity significantly increased from childhood through to the elderly group (ANOVA, $p < 1 \times 10^{-14}$). In contrast, the abundance of lactate-producing bacteria, key early-life colonizers, showed a consistent decrease with age ($p = 2.3 \times 10^{-57}$). The abundance of butyrate-producing bacteria peaked significantly in young and middle-aged adults ($p = 1.29 \times 10^{-12}$). The potential risk bacteria exhibited a U-shaped distribution, with the highest levels observed in children and the elderly ($p = 5.12 \times 10^{-10}$). Although the effect size was small ($R^2 = 0.0046$), a significant shift in the microbial community composition from childhood to old age was evident ($p = 0.001$, PERMANOVA; Figure 1B).

To identify the specific microbial taxa driving these age-related shifts, we analyzed differential abundances (Figure 1C,D). At the phylum level, *Actinobacteria*, which includes the prominent early-life genus *Bifidobacterium*, significantly decreased with age (Kruskal-Wallis with Dunn's test, q -value < 0.05). In contrast, *Verrucomicrobia* exhibited a U-shaped trend, with its lowest abundance in young adults (q -value < 0.05). At the genus level, these dynamics were even more pronounced. The abundance of

Bifidobacterium was highest in children and declined thereafter (q -value < 0.05), consistent with its role as a key infant colonizer. Conversely, genera such as *Oscillospira* and *Parabacteroides* steadily increased with age, peaking in the elderly group (q -value < 0.05). Other taxa showed more complex, non-linear patterns; for instance, *Prevotella* peaked in young adults, *Akkermansia* was lowest in the young and middle-aged groups before rising again in the elderly, while *Blautia* and *Ruminococcus* were most abundant in the teen and young adult groups before declining in later life (both q -value < 0.05).

3.2. Biological Sex Drives Distinct Gut Microbial Profiles

We next investigated sex-based differences in the gut microbiome (Figures 2A and S3, Supplementary Table S6). We found no significant difference in alpha diversity between males and females in our cohort (Figure 2A; t -tests and Benjamini-Hochberg correction, q -value > 0.05). However, the abundance of potential risk bacteria was significantly higher in females, while males showed higher abundances of both butyrate-producing and lactate-producing bacterial guilds (q -value < 0.01).

A PERMANOVA on beta diversity revealed a small but highly significant separation between the male and female microbiomes ($R^2 = 0.0059$; p -value = 0.001). To identify the specific taxa responsible for this separation, we performed differential abundance testing at the phylum and genus levels (Figure 2B,C). At the phylum level, females had a higher abundance of *Bacteroidetes* and *Verrucomicrobia*, whereas males were enriched in *Firmicutes* and *Actinobacteria* (q -value < 0.01). These phylum-level differences were driven by specific shifts at the genus level. For example, the higher abundance of *Bacteroidetes* in females was largely attributable to enrichments in the genera *Bacteroides* and *Parabacteroides*. Similarly, *Akkermansia* (the primary genus of *Verrucomicrobia*) was also significantly more abundant in females. Conversely, key beneficial genera such as *Faecalibacterium* and *Coproccoccus* were more abundant in males, contributing to their higher levels of butyrate-producing bacteria. We also noted that the abundance of *Prevotella* and *Bifidobacterium* was significantly higher in males (q -value < 0.01).

In summary, females harbor a higher abundance of several prominent genera including *Bacteroides* and *Akkermansia* along with a higher potential risk bacteria load, while males are enriched in key butyrate-producing taxa.

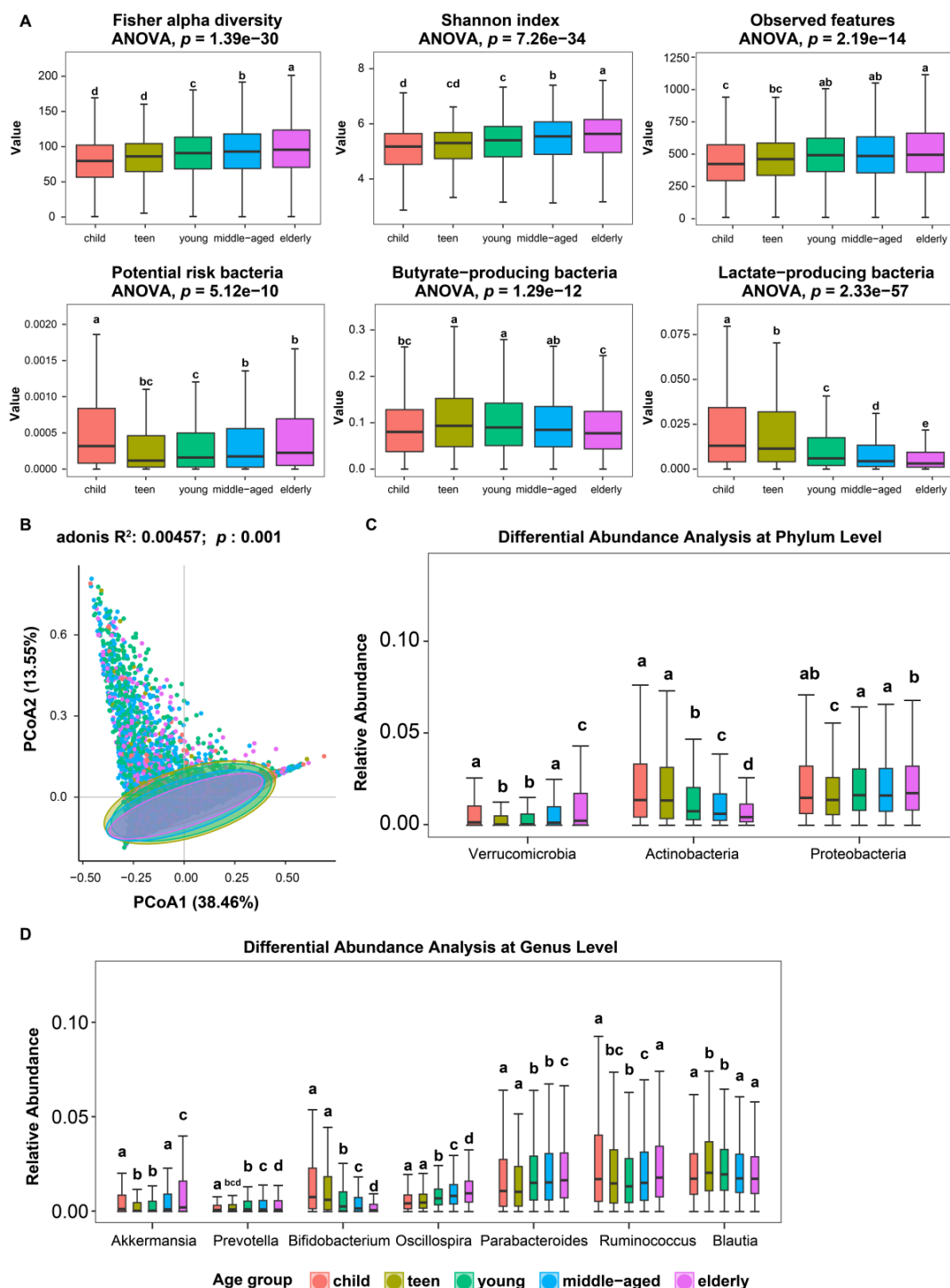


Figure 1. The Human Lifespan Shapes Distinct Gut Microbial Trajectories. **(A)** Boxplots of key microbial indices across five age groups: child ($n = 802$), teen ($n = 308$), young ($n = 3699$), middle-aged ($n = 5044$), and elderly ($n = 3278$). Indices include three alpha diversity metrics, the relative abundance of potential risk bacteria, and the relative abundances of butyrate- and lactate-producing bacteria. The line within each box represents the median, the box bounds represent the interquartile range (IQR), and the whiskers extend to 1.5 times the IQR. Statistical significance was assessed using a one-way ANOVA, with overall p -values shown for each panel. Letters above the boxes denote groups that are significantly different from each other based on a post-hoc Tukey's HSD test ($p < 0.05$). **(B)** Principal Coordinate Analysis (PCoA) plot based on Bray-Curtis dissimilarity, illustrating the overall shift in microbial community structure with age. Each point represents an individual sample, colored by age group. Ellipses represent the 95% confidence interval for each group's centroid. The overall separation between groups is statistically significant (PERMANOVA, $R^2 = 0.0046$, $p = 0.001$). **(C,D)** Differential abundance of the most prevalent microbial phyla **(C)** and genera **(D)** across the five age groups. The relative abundance of each taxon is shown. Statistical significance was determined using a Kruskal-Wallis test followed by a Dunn's post-hoc test for pairwise comparisons, with Benjamini-Hochberg correction for multiple tests. Within each taxon plot, groups that do not share a letter are significantly different ($q < 0.05$).

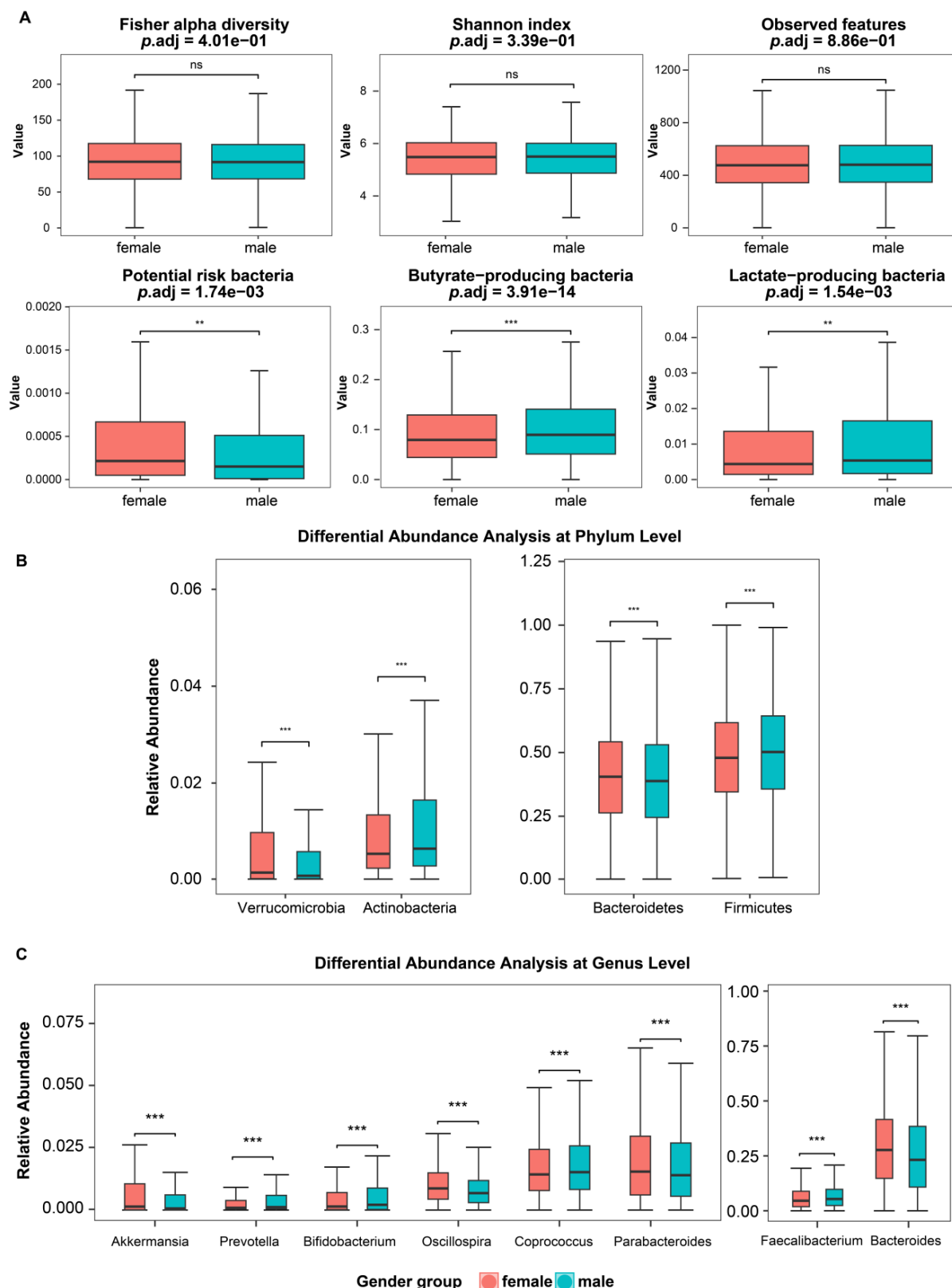


Figure 2. Biological Sex Drives Distinct Gut Microbial Profiles. (A) Comparison of key microbial indices between female ($n = 6722$) and male ($n = 6072$) participants. Indices include three alpha diversity metrics, the relative abundance of potential risk bacteria, and the relative abundances of butyrate- and lactate-producing bacteria. The line within each box represents the median, the box bounds represent the interquartile range (IQR), and the whiskers extend to 1.5 times the IQR. Statistical significance was assessed using independent samples t -tests with Benjamini-Hochberg correction for multiple comparisons. Adjusted p -values (p_{adj}) are shown for each comparison. (B,C) Differential abundance of the most prevalent microbial phyla (B) and genera (C) between female and male participants. The relative abundance of each taxon is shown. Statistical significance was determined using a Wilcoxon rank-sum test with Benjamini-Hochberg FDR correction. Asterisks denote the level of significance: ** $q < 0.01$, *** $q < 0.001$; ns, not significant.

3.3. Age Stratifies the Gut Microbiome's Response to Environmental and Lifestyle Factors

We next sought to determine whether the influence of key environmental and personal factors was also age-dependent. We performed a systematic analysis, assessing the association of 86 variables with 10 microbial indices across our five age strata (Figures 3 and S4).

The impact of diet was most potent during the years of childhood. Consumption of healthy foods like fruit and whole grains was associated with increased alpha diversity, but this effect was most pronounced in children (linear regression analysis, Adjusted $R^2 = [0.0013, 0.018]$, q -value < 0.001 ; Figure 3A). In contrast, these dietary factors exerted their strongest influence on overall community structure (beta diversity) in the elderly population ($R^2 = [0.006, 0.01]$, q -value < 0.001). This suggests that dietary interventions may have different functional consequences depending on life stage.

Antibiotic use, a major medical exposure, was associated with reduced alpha diversity and altered microbial community structure across all age groups ($R^2 = [-0.017, 0.024]$, q -value < 0.01 ; Figure 3B). Exposure to various airborne POPs had clear age-biased effects. For instance, Endosulfan's impact on observed features was strongest in children (Adjusted $R^2 = 0.032$, q -value < 0.001), whereas Dieldrin, Chlordane, PCB and dlPCB's influence was greatest in elderly individuals ($R^2 = [0.004, 0.028]$, q -value < 0.001 ; Figure 3C). Several POPs, notably AlphaHCH, Endosulfan, and Heptachlor, were associated with an increase in the abundance of potential risk bacteria, an effect seen almost exclusively in middle-aged and elderly individuals ($R^2 = [0.003, 0.006]$, q -value < 0.001). While most air pollutants like PM_{2.5} were associated with a decrease in microbial diversity ($R^2 = [-0.011, -0.006]$, q -value < 0.001), O₃ exposure was linked to an increase in microbial diversity (Adjusted $R^2 = [0, 0.002]$, q -value < 0.05), highlighting the complex and sometimes opposing effects of environmental factors.

Finally, lifestyle factors also showed age-dependent associations (Figure 3E). The positive effect of exercise frequency on alpha diversity was most significant in children ($R^2 = [0.009, 0.020]$, q -value < 0.05), perhaps reflecting its role in shaping the developing microbiome. In contrast, factors representing novel microbial exposure, such as recent travel and frequency of pool use, had the most prominent impact on the gut microbiome of the elderly (Adjusted $R^2 = [0.003, 0.013]$, q -value < 0.01), suggesting a less resilient microbial community that is more susceptible to perturbation by external environmental inputs.

3.4. Sex Stratifies the Gut Microbiome's Response to Environmental and Lifestyle Factors

Beyond age, we investigated whether biological sex acts as a key modifier of the gut microbiome's response to environmental and personal factors (Figures 4 and S5).

Dietary habits were a prominent example of these sex-specific effects. While consumption of fruits and whole grains was associated with increased alpha diversity in both sexes ($R^2 = [0.001, 0.005]$, q -value < 0.05), the beneficial effect of a healthy diet in reducing the potential risk bacteria was significant only in females ($R^2 = [-0.003, -0.002]$, q -value < 0.01) (Figure 4A).

We observed even stronger sex-specific responses to chemical exposures. The effect of antibiotic use on reducing alpha diversity, for instance, was greater in females than in males (female, $R^2 = [-0.018, -0.004]$, male: $R^2 = [-0.007, -0.004]$, q -value < 0.001 ; Figure 4B). While various POPs were associated with increased alpha diversity in both sexes, the magnitude of this effect for the pollutant Dieldrin was more than double in females compared to males (female $R^2 = [0.021, 0.022]$, male $R^2 = [0.010, 0.012]$, q -value < 0.001), indicating a heightened microbial sensitivity to this specific toxicant in females (Figure 4C). In contrast, air pollutants like CO and O₃ had a more pronounced effect on the beta diversity of females (female $R^2 = [-0.004, 0.007]$, q -value < 0.001 , male $R^2 = [-0.002, 0.001]$, q -value < 0.001 ; Figure 4D).

Lifestyle factors also exhibited sex-divergent associations. Notably, exercise frequency was associated with a significant increase in alpha diversity only in females ($R^2 = [0.004, 0.008]$, q -value < 0.001), whereas factors related to travel and swimming were more strongly associated with increased alpha diversity in males ($R^2 = [0.004, 0.007]$, q -value < 0.001 ; Figure 4E).

3.5. Gut Microbiome's Potential Role in Mediating Health Risks is Stratified by Age and Sex

Finally, we used mediation analysis to test our central hypothesis: the gut microbiome's potential role in linking environmental exposures to health outcomes is dependent on age and sex. Our systematic analysis identified a few thousands potential mediation effects across multiple health outcomes (Supplementary Tables S7 and S8). Here, we highlight three of the most salient examples that illustrate the distinct ways in which age and sex stratify these risk pathways (Figure 5).

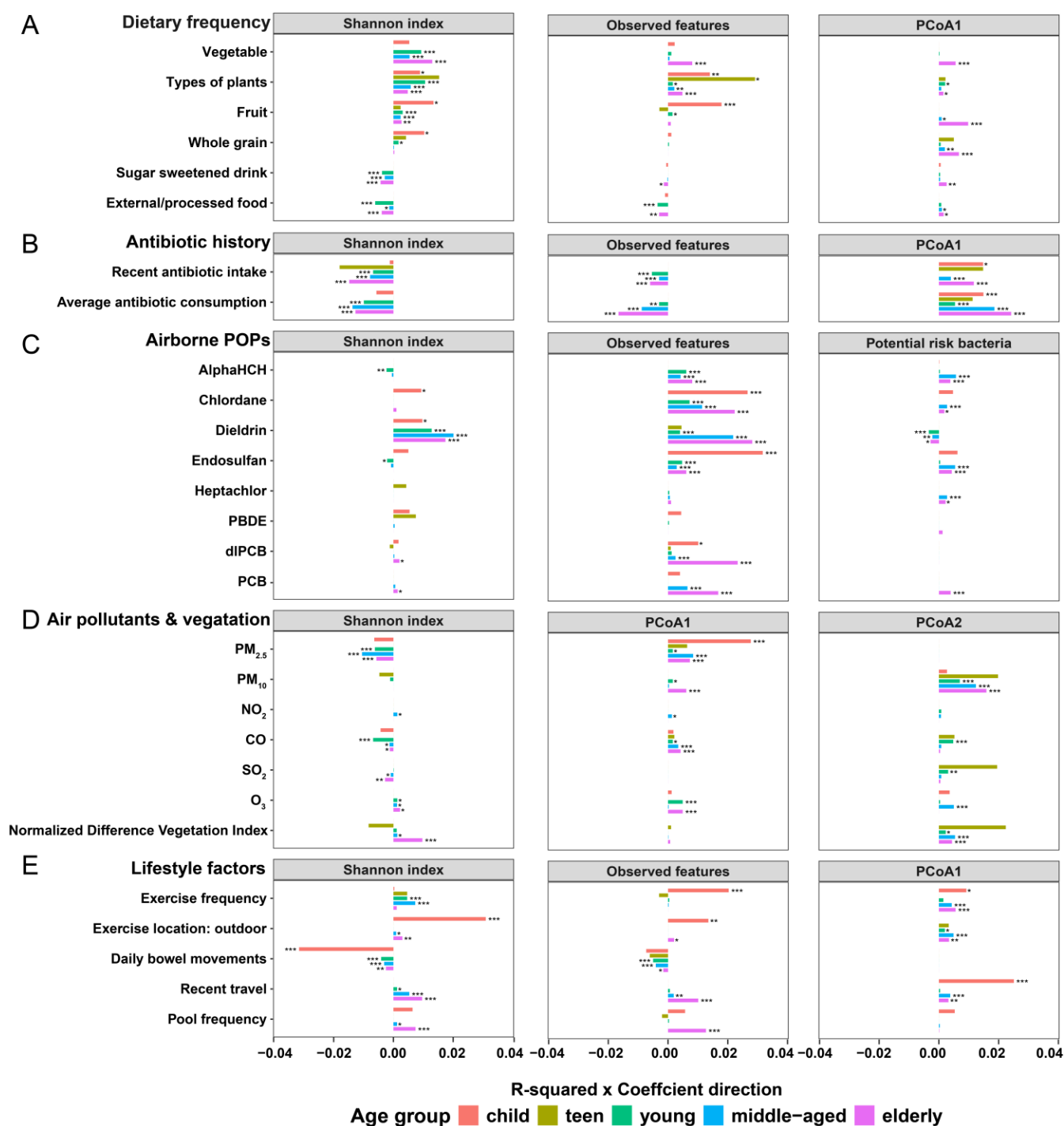


Figure 3. Age Stratifies the Gut Microbiome's Response to Environmental and Lifestyle Factors. This figure illustrates the age-stratified associations between univariate linear regressions performed separately for each of the five age groups. The length of each bar represents the proportion of variance in the microbial index explained by the factor (Adjusted R^2), and the direction of the bar indicates a positive (right) or negative (left) association. (A) Dietary Frequency: Associations between dietary habits and three microbial indices. (B) Antibiotic History: Associations between antibiotic use and three microbial indices. (C) Airborne POPs: Associations between exposure to specific Persistent Organic Pollutants (POPs) and three microbial indices. (D) Air Pollutants & Vegetation: Associations between air pollutants/NDVI and three microbial indices. (E) Lifestyle Factors: Associations between lifestyle activities and three microbial indices. Asterisks denote the statistical significance of each association after Benjamini-Hochberg correction for multiple tests: * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$.

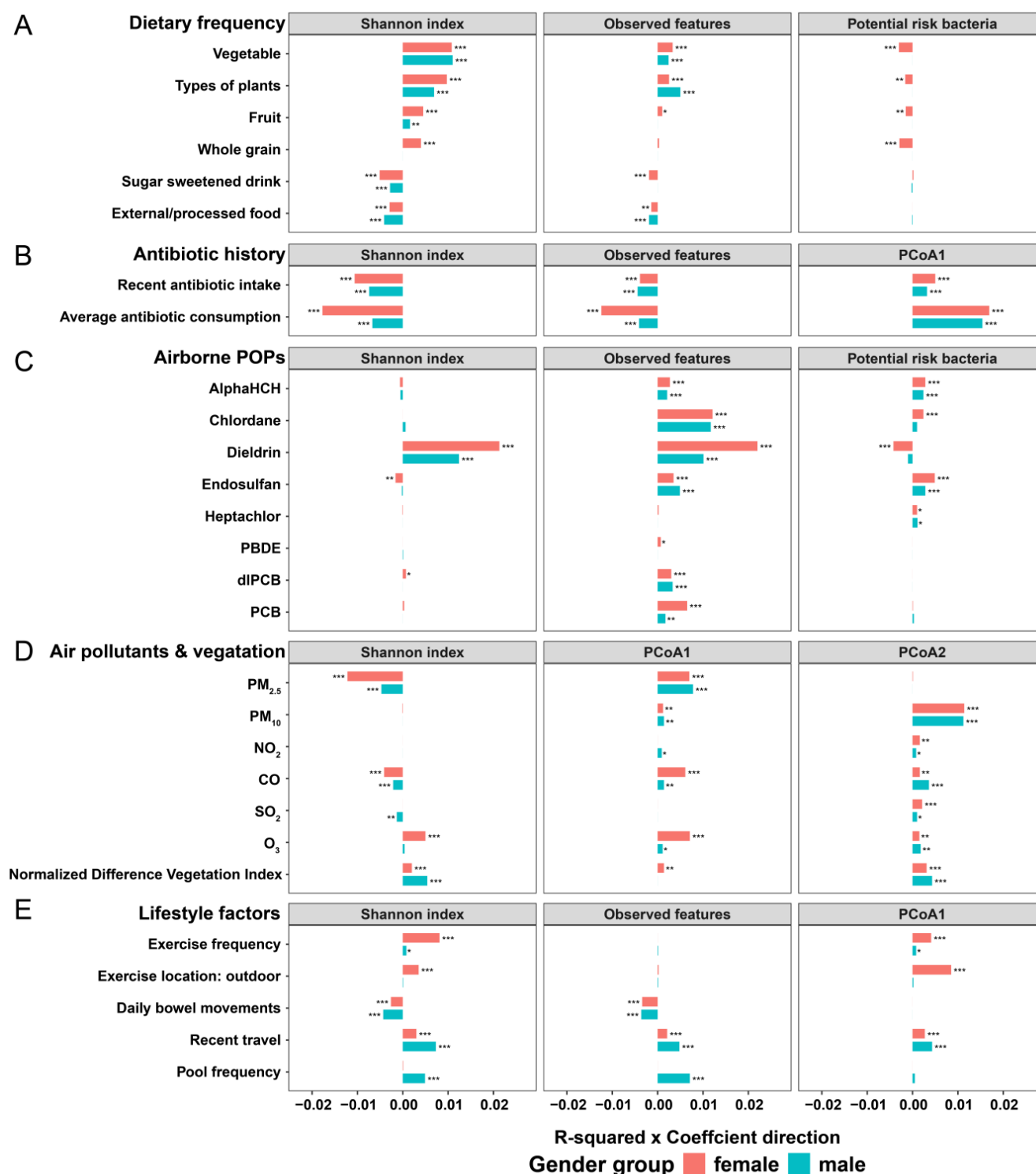


Figure 4. Sex Stratifies the Gut Microbiome's Response to Environmental and Lifestyle Factors. This figure illustrates the sex-stratified associations between various environmental/personal factors (y-axis) and select gut microbial indices (panel columns). Bar charts display the results of univariate linear regressions performed separately for female and male participants. The length of each bar represents the proportion of variance in the microbial index explained by the factor (Adjusted R^2), and the direction of the bar indicates a positive (right) or negative (left) association. (A) Dietary Frequency: Associations between dietary habits and three microbial indices, stratified by sex. (B) Antibiotic History: Associations between antibiotic use and three microbial indices, stratified by sex. (C) Airborne POPs: Associations between exposure to specific Persistent Organic Pollutants (POPs) and three microbial indices, stratified by sex. (D) Air Pollutants & Vegetation: Associations between air pollutants/NDVI and three microbial indices, stratified by sex. (E) Lifestyle Factors: Associations between lifestyle activities and three microbial indices, stratified by sex. Asterisks denote the statistical significance of each association after Benjamini-Hochberg correction for multiple tests: * $q < 0.05$, ** $q < 0.01$, *** $q < 0.001$.

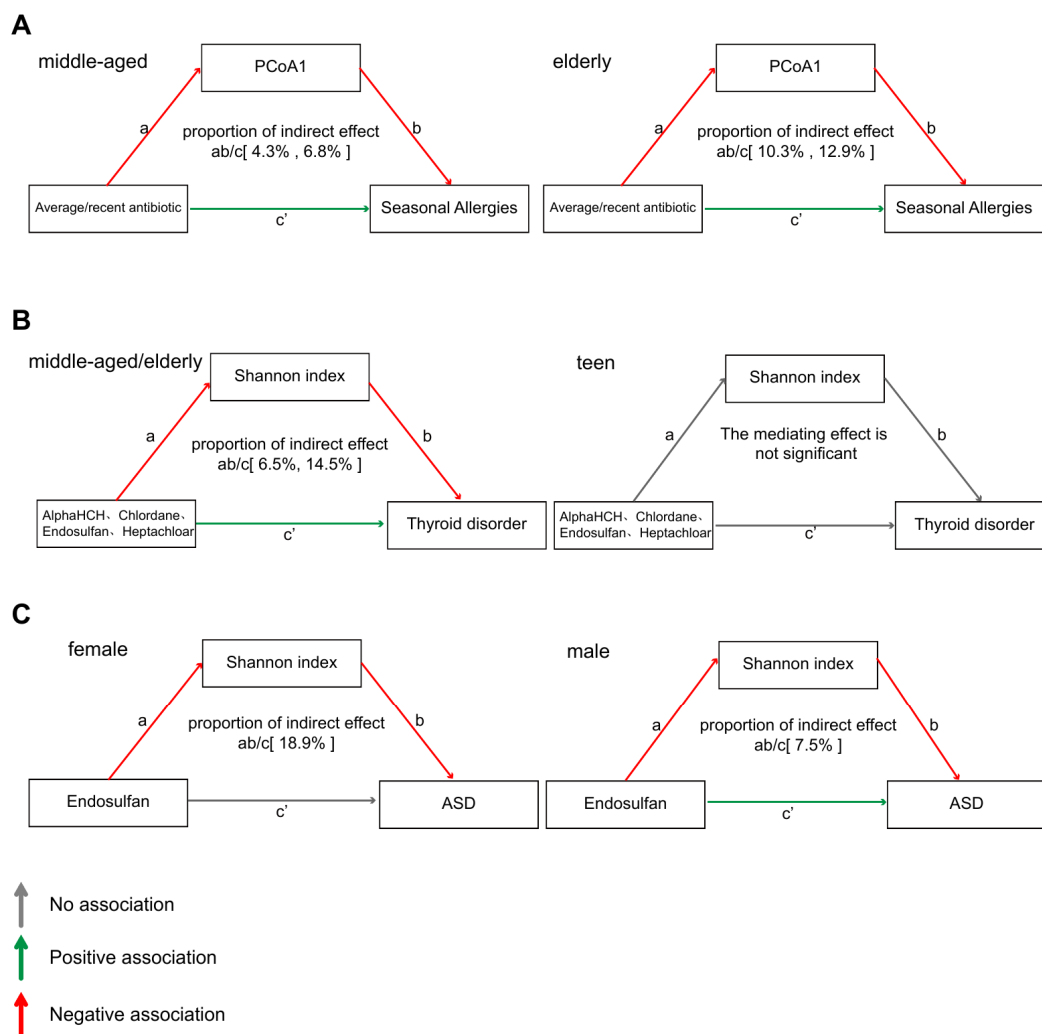


Figure 5. The Gut Microbiome's Role as a Mediator of Environmental Health Risks is Stratified by Age and Sex. Mediation models illustrating how the effect of an environmental exposure (left box) on a health outcome (right box) is mediated by a microbial index (top box). Arrows represent the direction of the association (pathways 'a', 'b', and 'c'). Green arrows indicate a positive association, red arrows indicate a negative association, and gray arrows indicate no significant association. The proportion of the total effect that is mediated by the indirect pathway is shown for each significant model. (A) Age-stratified mediation of antibiotic use and seasonal allergy risk. The indirect pathway, mediated by changes in community structure (PCoA1), is significantly stronger in the elderly compared to the middle-aged group. (B) Age-stratified mediation of POP exposure and thyroid disorder risk. The indirect pathway, mediated by a decrease in Shannon diversity, is significant in middle-aged and elderly adults but not in teens. (C) Sex-stratified mediation of Endosulfan exposure and Autism Spectrum Disorder (ASD) risk. The risk pathway is fundamentally different between the sexes. In males, the direct effect (path c') is the dominant risk pathway. In females, the indirect effect, mediated by a decrease in Shannon diversity, is the prominent pathway.

Recent antibiotic use was associated with an increased risk of seasonal allergies across all age groups (Total Effect $c = 0.103 \sim 0.123$, $p < 0.05$). However, the proportion of this risk that was indirectly mediated by the microbiome (via changes in PCoA1) was significantly larger in the elderly (10.3–12.9%) compared to the middle-aged group (4.3–6.8%), suggesting a distinct age-associated disparity in the microbiome's role in this pathway (Figure 5A). Similarly, the pathway linking exposure to POPs with thyroid disorder risk was significant in middle-aged and elderly adults but not in teens (Figure 5B). In older adults, POP exposure was

associated with increased thyroid disorder risk (Total Effect $c = -0.256 \sim 0.369$, $p < 0.05$), operating both directly and indirectly, with the indirect path operating through a significant reduction in Shannon diversity.

One example from our sex-specific analysis linking the pollutant Endosulfan to the risk of Autism Spectrum Disorder (ASD) (Figure 5C). This risk pathway appeared fundamentally different between males and females. In males, Endosulfan exposure was associated with a significant total increase in ASD risk (Total Effect $c = 0.254 \sim 0.271$, $p < 0.05$) and a significant direct increase ($c' = 0.253$, 95% CI [0.097, 0.417]), with a smaller, but still

significant, indirect effect mediated by the microbiome. In contrast, the direct effect of Endosulfan on ASD risk was not significant in females ($c' = 0.197$, 95% CI $[-0.046, 0.456]$). For females, the primary link between the pollutant and the outcome appeared to be the indirect pathway operating through a reduction in Shannon diversity. This suggests that the biological mechanism of Endosulfan-associated ASD risk may be sex-dependent, with males potentially being more vulnerable to a direct toxicological effect, while females are more susceptible to a microbiota-mediated disruption.

Notably, these stratified pathways align with well-known epidemiological patterns. The finding that a microbiome-mediated risk for thyroid dysfunction is prominent in older adults is consistent with the increasing prevalence of thyroid disease with age [14]. The sex-dependent risk pathway for ASD—with males more vulnerable to a direct toxicological effect—is consistent with the well-documented male bias in ASD diagnoses [15,16]. This demonstrates how a stratified analysis can provide mechanistic insights for understanding major demographic disparities in human health.

4. Discussion

In this study, we leveraged a large-scale, population-based cohort to test the hypothesis that the gut microbiome's potential role in mediating environmental health risks is stratified by age and sex. Our findings support this hypothesis, revealing distinct microbial response patterns and risk pathways in different demographic subgroups and providing a data-driven framework for understanding major disparities in human health.

4.1. Strengths and Limitations of the Study

A primary strength of this work is its systematic, hypothesis-driven investigation into the interactive effects of age, sex, environment, and the microbiome on health. While previous studies have examined these factors in isolation, our work is, to our knowledge, the first to use a formal mediation framework to dissect how these variables operate across dozens of health outcomes. By integrating a large, global cohort with extensive environmental and geospatial databases, we created an analytical resource that enabled this multi-dimensional analysis. The identification of specific, testable, and clinically relevant risk pathways is a key novelty that moves beyond simple association to generate concrete hypotheses for future mechanistic studies.

We acknowledge several limitations in our study. First, the cross-sectional nature of the data cannot definitively establish causality, although our formal mediation analysis provides strong statistical evidence consistent with the proposed pathways. Longitudinal studies that track individuals over time are an essential next step to confirm our findings. Second, our exposure

assessment relied on geolocational data aggregated at the city level. We acknowledge that this approach introduces exposure misclassification (ecological fallacy), as it cannot capture significant intra-city spatial heterogeneity driven by factors such as proximity to major roadways, industrial zones, or indoor microenvironments. Statistically, this lack of granularity serves as a source of noise that likely leads to non-differential misclassification, thereby attenuating the observed effect sizes toward the null (regression dilution bias). Consequently, the associations reported here may represent conservative estimates of the true biological impact. While we utilized a three-year average to model chronic “urban background” exposure rather than acute personal variations, future studies incorporating high-resolution personal monitoring are essential to capture the full magnitude of these environment-microbiome interactions. Similarly, our mycotoxin model estimates exposure potential rather than precise dosage, as biological validation (e.g., biomarkers) was unavailable. This approach relies on regional averages that may overlook local sourcing variations and utilizes self-reported dietary data subject to recall bias. Therefore, these associations should be interpreted as probabilistic risk scores derived from geography and diet, rather than confirmed toxicological intake. Third, our definition of functional guilds (e.g., butyrate producers) relies on taxonomic identity as a proxy for metabolic potential. As 16S rRNA sequencing cannot resolve strain-specific gene content or expression, these associations reflect the abundance of taxa capable of these functions, rather than confirmed metabolite production. Fourth, all health outcomes were self-reported, which may be subject to recall bias or misclassification. Given the complexity of conditions like ASD and thyroid disorders, we acknowledge that self-reported diagnoses lack clinical validation. Consequently, the health-associated findings in this study should be viewed as exploratory and hypothesis-generating, requiring validation in clinically phenotyped cohorts. However, the use of standardized questionnaire data from a large cohort like the AGP allows for the discovery of strong, population-level signals that can guide more targeted clinical research. Fifth, the generalizability of our findings may be limited, as the American Gut Project cohort is a self-selected group of participants, predominantly from North America and Europe, who are likely more health-conscious than the general population. Expanding these analyses to include more diverse global populations will be crucial to determine the generalizability of these age- and sex-specific patterns. Despite these limitations, a primary strength of this work remains its systematic investigation into the interactive effects of age, sex, environment, and the microbiome across nearly 14,000 individuals, providing novel, testable hypotheses for future mechanistic studies. Finally, we acknowledge that the effect sizes (R^2) observed for individual environmental

factors were generally small (typically explaining < 1% of variance). This is a common feature of population-scale microbiome studies, reflecting the immense complexity of the ecosystem where thousands of variables—from host genetics to micro-dietary components—contribute to community structure. However, given the ubiquity of environmental exposures like air pollution and POPs, even small shifts in the microbial ‘baseline’ of a population can have meaningful cumulative impacts on public health resilience.

4.2. Age- and Sex-Related Trajectories of Key Microbial Taxa and Functional Groups

Previous studies often reported a decline in diversity with aging [17,18]. The decreased alpha diversity is linked to factors like reduced dietary breadth or polypharmacy in some elderly cohorts. Our observation of sustained high alpha diversity into older adulthood aligns with reports from exceptionally healthy aging populations [19,20]. To investigate potential selection bias, we compared the body mass index (BMI) of our elderly US participants ($n = 3278$) against national averages. The mean BMI in our elderly cohort was 24.1, significantly lower than the national average of ~ 29.1 reported for this age group in the NHANES 2015–2018 dataset [21]. This suggests that the AGP elderly cohort represents a ‘super-ager’ or ‘healthy survivor’ phenotype. Consequently, our findings support the hypothesis that maintained ecosystem resilience, rather than uniform decline, characterizes the microbiome of healthy aging.

We observed a U-shaped abundance of potential risk bacteria, peaking in childhood and late adulthood. This likely reflects the immature pediatric immune system and microbiota [22,23] versus age-related immunosenescence and physiological changes in the elderly [23,24], highlighting windows of vulnerability. Concurrently, butyrate-producing bacteria abundance peaked in adulthood, consistent with microbiota maturation for fiber fermentation [24] and a subsequent decline often seen in older age linked to dietary shifts and inflammation [25,26]. Furthermore, a decrease in early-life colonizers like lactate-producing bacteria with age reflects their foundational role in immune programming [27,28] before giving up dominance to a more complex adult microbiota [29]. Collectively, these non-linear dynamics underscore that the aging gut microbiome is not monolithic. Different life stages present unique microbial ecological landscapes and vulnerabilities, suggesting age-tailored strategies may be needed to foster or restore a health-associated gut community.

Our investigation into sex-specific gut microbiota profiles also revealed subtle yet significant distinctions. A key finding was the significantly higher abundance of intestinal potential risk bacteria in the female group. Conversely, males exhibited greater levels of beneficial butyrate-producing and lactate-producing bacteria. These

compositional differences contributed to a statistically significant, albeit small, divergence in overall gut microbial community structure between sexes. The observation of increased potential risk bacteria in females warrants further investigation, as it could suggest a sex-specific predisposition to harboring opportunistic pathobionts, potentially influenced by hormonal fluctuations impacting the gut environment or distinct immune responses [29]. The higher prevalence of butyrate and lactate producers in males is also noteworthy, as these bacteria are crucial for colonic health, energy metabolism, and immune modulation [30–32]. These consistent microbial signatures underscore the importance of considering sex as a biological variable in gut microbiome research and its potential implications for sex-specific health trajectories or disease susceptibilities.

4.3. Persistent Organic Pollutants: Age-Specific and Sex-Divergent Modulators of Gut Microbiota and Mediators of Disease Risk

Our findings suggest POPs are significant disruptors of gut microbial homeostasis across age groups, impacting diversity and enriching for potential risk bacteria. This generalized susceptibility was nuanced by age-specific responses to individual POPs; for example, Dieldrin, Chlordane, PCB, and dlPCB more strongly affected middle-aged/elderly groups, while Endosulfan’s impact was more pronounced in children. The observed co-occurrence of increased alpha diversity with elevated risk bacteria following some POP exposures suggests a dysbiotic proliferation rather than healthy diversification, warranting cautious interpretation [33]. Sex further modulated these interactions, with females exhibiting heightened microbial sensitivity to Dieldrin, potentially due to sex-specific physiological factors influencing POP handling [34,35].

Crucially, mediation analyses linked these POP-induced microbial shifts to downstream health risks in an age- and sex-dependent manner, providing potential mechanistic insights consistent with well-established epidemiological patterns. For thyroid disorders, POP exposure increased risk in middle-aged and elderly individuals through both direct effects and indirectly by reducing Shannon diversity. This age-dependent risk pathway is highly consistent with the known increase in thyroid disease prevalence with age [14], and suggests a complex interplay where gut dysbiosis contributes to POP-associated thyroid pathology [36–39]. The Endosulfan-ASD risk association also revealed striking sex-specific mediation. This finding directly addresses the well-documented 3:1 male-to-female bias in ASD diagnoses [15]. Our model suggests a potential explanation for this disparity: males showed a more prominent direct risk increase, with microbial disruption playing a partial mediating role, while in females, the

indirect, microbiota-mediated pathway via reduced Shannon diversity appeared more dominant. These distinct pathways highlight the necessity for sex-stratified risk assessment of environmental toxicants and implicate the gut microbiome as a sex-specific mediator and potential therapeutic avenue [40,41].

4.4. Age and Sex Modulate Gut Microbiome Responses to Diet and Lifestyle Factors

Our analysis reveals that the impact of dietary habits, vitamin intake, and lifestyle choices on the gut microbiome is not uniform, but rather modulated by age and sex. While the benefits of diets rich in fruits, vegetables, and whole grains for gut microbial diversity are well-established [42], our findings highlight specific windows of heightened impact. Notably, fruit and whole grain consumption demonstrated a more pronounced positive effect on alpha diversity in children and on beta diversity in the elderly. This suggests that early-life dietary interventions emphasizing these foods could be particularly effective in establishing a diverse foundational microbiome, while in later life, they may play a crucial role in maintaining community stability and structure against age-related drift [43–45].

Lifestyle factors, including exercise, travel, and pool frequency, also exerted age-dependent effects. While exercise generally benefited microbial diversity, its impact was more significant in children. Conversely, recent travel and pool frequency had a more prominent influence on the gut microbiota of the elderly. The heightened effect of exercise in children may reflect its role in shaping a still-developing microbiota through mechanisms like altered gut motility or direct microbial exchange [46]. For the elderly, increased microbial shifts due to travel or pool exposure could indicate either a greater susceptibility to environmental microbial introduction due to a less resilient baseline microbiota, or perhaps a more pronounced beneficial diversification from novel exposures if managed appropriately [47–49].

4.5. Specific Microbial Trajectories with Age and Sex

Our findings of age-dependent shifts in specific taxa align with and extend previous observations of microbial succession across the lifespan. The “high in childhood, lower in adulthood” trajectory we observed for *Bifidobacterium* is a hallmark of the maturing gut, consistent with its role as a key early-life colonizer fueled by milk oligosaccharides [6,50]. In contrast, the steady increase of butyrate-producers like *Oscillospira* into old age supports models of a healthy aging microbiome [51]. The corresponding decline we noted in *Bacteroides* in the elderly may reflect common dietary shifts in this population towards lower-fat diets, as *Bacteroides* growth can be inhibited by the accumulation of secondary bile acids associated with such diets [52,53].

Our analysis also revealed distinct sex-specific microbial signatures, which likely arise from a complex interplay of hormones, diet, and immunity. The enrichment of *Bacteroides* and *Akkermansia* in females in our cohort is consistent with studies linking these genera to estrogen levels and fiber metabolism [54]. Conversely, the higher abundance of certain *Firmicutes* genera like *Faecalibacterium* in males may reflect sex-based differences in diet or energy metabolism [55]. The observed sex bias for *Prevotella*, which we found to be more abundant in males, is a topic of ongoing debate in the literature. Some studies have reported a similar male bias, while others associate *Prevotella* with female cohorts; these discrepancies are often attributed to its strong dependence on dietary fiber intake, which can overwhelm hormonal influences [56,57]. Therefore, the specific direction of the *Prevotella* sex bias can appear inconsistent across different study populations, likely reflecting cohort-specific dietary patterns rather than a universal hormonal effect.

4.6. Implications for Stratified Medicine

The findings from this study have significant implications for the future of preventive and personalized medicine. By demonstrating that the microbiome’s potential role as a mediator of environmental risk is fundamentally stratified by age and sex, our work argues against a “one-size-fits-all” model of disease prevention. Instead, it provides a data-driven rationale for stratified interventions. For example, the sex-dependent pathway we identified in ASD suggests that therapeutic strategies might need to be sex-specific: a microbiome-targeted therapy (e.g., prebiotics or probiotics) could be more effective for females, whereas strategies to mitigate direct neurotoxicity might be more critical for males. Similarly, public health guidelines for pollutant exposure could be tailored, with a greater focus on protecting the elderly, in whom the microbiome appears to be a more significant mediator of toxicant-induced pathology. Ultimately, this work provides a foundational map for developing more precise, demographically aware strategies—from sex-informed diagnostics to age-tailored interventions—to promote human health in a complex world.

Supplementary Materials

The additional data and information can be downloaded at: <https://media.sciltp.com/articles/others/2608061416436311/GES-26010022-SM.pdf>. Figure S1: Schematic Overview of the Data Integration and Analysis Workflow; Figure S2: Decision Framework for the Interpretation of Mediation Analysis Results; Figure S3: Age- and Sex-Based Differences in Community-Wide Microbial Ratios and Beta Diversity Coordinates; Figure S4: Age-Stratified Effects of Alcohol and Vitamin Consumption on the Gut Microbiome; Figure S5: Sex-Stratified Effects of Alcohol and Vitamin Consumption on the Gut Microbiome.

Table S1: Data Variable Sources; Table S2: The frequency of environmental/individual characteristics of each age group; Table S3: The frequency of environmental/individual characteristics of each sex group; Table S4: Classification of Mediation Effects; Table S5: Comparative results of gut microbial indices in different age groups; Table S6: Comparative results of gut microbial indices in different sex groups. The q-value is adjusted p-values (FDR); Table S7: Age group mediation effect analysis; Table S8: Sex group mediation effect analysis.

Author Contributions

J.O.: conceptualization, formal analysis, writing—original draft preparation; Y.T. and X.W.: data curation, methodology; Y.Y.: data curation, validation; X.F. and Y.S.: supervision, writing—review and editing, funding acquisition. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Ethical review and approval were waived for this study, as it represents a secondary analysis of a fully de-identified, publicly available dataset from the American Gut Project (AGP). The original AGP study protocol was approved by the Institutional Review Board of the University of California San Diego.

Informed Consent Statement

Informed consent was obtained from all subjects involved in the original American Gut Project (AGP) study by the primary investigators. As this current study exclusively utilized fully anonymized, publicly available secondary data, obtaining new informed consent was not applicable.

Data Availability Statement

The 16S rRNA sequencing data and associated metadata analyzed in this study were sourced from the American Gut Project and are publicly available at the project's FTP site: <http://ftp.microbio.me/AmericanGut/latest/>. The public environmental and geospatial databases used are detailed in the Supplementary Methods (Supplementary Tables S1–S3). The R scripts used for analysis are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflict of interest.

Use of AI and AI-Assisted Technologies

During the preparation of this work, the authors used Gemini to improve the English language and

readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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