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# Sick Individuals and Sick (Microbial) Populations: Challenges in Epidemiology and the Microbiome

Audrey Renson,<sup>1</sup> Pamela Herd,<sup>2</sup> and Jennifer B. Dowd<sup>3,4</sup>

<sup>1</sup>Department of Epidemiology, Gillings School of Global Public Health, University of North Carolina, Chapel Hill, North Carolina 27599, USA; email: arenson@live.unc.edu

<sup>2</sup>McCourt School of Public Policy, Georgetown University, Washington, DC 20057, USA; email: ph627@georgetown.edu

<sup>3</sup>Department of Global Health and Social Medicine, King's College London, London WC2B 4BG, United Kingdom; email: jennifer.dowd@kcl.ac.uk

<sup>4</sup>Current affiliation: Leverhulme Center for Demographic Science, University of Oxford, Oxford OX1 1JD, United Kingdom; email: jennifer.dowd@sociology.ox.ac.uk

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## Keywords

microbiome, microbiota, SES, race/ethnicity, social, metabolic,  
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## Abstract

The human microbiome represents a new frontier in understanding the biology of human health. While epidemiology in this area is still in its infancy, its scope will likely expand dramatically over the coming years. To rise to the challenge, we argue that epidemiology should capitalize on its population perspective as a critical complement to molecular microbiome research, allowing for the illumination of contextual mechanisms that may vary more across populations rather than among individuals. We first briefly review current research on social context and the gut microbiome, focusing specifically on socioeconomic status (SES) and race/ethnicity. Next, we reflect on the current state of microbiome epidemiology through the lens of one specific area, the association of the gut microbiome and metabolic disorders. We identify key methodological shortcomings of current epidemiological research in this area, including extensive selection bias, the use of noncompositionally robust measures, and a lack of attention to social factors as confounders or effect modifiers.

## INTRODUCTION

Since the first publication of results from the Human Microbiome Project (HMP) in 2012 (61), research on the microbiome—the trillions of microorganisms that inhabit the human body and their genes—has grown exponentially,<sup>1</sup> generating new knowledge on everything from how diet affects the microbiome to how the microbiome may influence the brain, behavior, and mental illness (4, 117). Robust evidence points to a stronger role for the “environment” in shaping the human gut microbiome relative to genetics (111), compelling researchers to better define and measure the environment to understand the role of the microbiome in human health and disease (53). Use of model systems such as germ-free mice allows strong causal inference on isolated aspects of microbiome biology, but analysis of human populations in their full complexity is necessary to move beyond general principles toward actionable knowledge (83). Thus, while it remains important to understand microbiome function at the molecular level with an eye toward novel prognostic and treatment breakthroughs, it is equally important to zoom out and consider a population perspective in microbiome research (109). A population perspective reminds us that individual-level determinants of the microbiome are not necessarily the same as those that explain differences across populations, especially those living within quite homogeneous environments with respect to geography, culture, and nutrition (109). Social and geographical contexts, for instance, are emerging both as crucial determinants of the microbiome itself and as modifiers of existing microbiome–health associations (29, 50). Epidemiology is well poised to make important contributions to the description of the microbiome across person, place, and time, as well as to the improvement of population-based research design and causal inference to understand how the microbiome impacts health and disease across the life course (108).

This review briefly summarizes current research on social context and the gut microbiome, focusing specifically on socioeconomic status (SES) and race/ethnicity. We then reflect on the current state of microbiome epidemiology through the lens of one specific area: the association of the gut microbiome and metabolic traits. As in any new research area spurred forward by new technology, each step forward often illuminates an even longer path toward actionable knowledge. Although epidemiological research on the microbiome is in its infancy, this review aims to raise awareness of key methodological challenges and the importance of the broader social and environmental contexts influencing both the microbiome and health.

### Social Context and the Microbiome

While providing much-needed novel description, the HMP project was designed to characterize the human microbiome for the first time only in a small group of healthy volunteers (61). An important next step has been to describe how the microbiome varies across more diverse populations and especially across characteristics known to have large associations with overall health and longevity such as SES and race/ethnicity.

### Socioeconomic Status and the Microbiome

Through pathways such as living conditions, psychosocial stress, and nutrition, it is likely that social conditions across the life course act to significantly shape environmental drivers of the gut microbiome (30, 53). We define SES as reflecting human capital and economic resources such as education, income and wealth, and occupation. Socioeconomic resources can also be

<sup>1</sup>From 43 papers in 2000 to 5,032 in 2018, via a PubMed search for “microbiota OR microbiome AND human,” on May 24, 2019.

conceptualized and measured at the neighborhood level. Though SES is frequently treated as a unitary concept in biomedical research, we encourage consideration of separable influences of socioeconomic indicators where possible to better understand the pathways and potential targets for intervention (52).

To date, we have identified two studies that specifically link varying socioeconomic conditions to alterations in the gut microbiome (10, 87). Miller et al. (87) related a composite indicator of neighborhood SES to the microbiota composition of 44 healthy volunteers in Chicago, Illinois, finding higher  $\alpha$ -diversity and greater abundance of *Bacteroides* with increased neighborhood SES. In a large sample of twins in the United Kingdom, Bowyer et al. (10) identified associations between individual and area-level income and diversity and relative abundance of operational taxonomic units (OTUs) in the gut microbiome. Community composition measured by Bray–Curtis dissimilarity was found to differ by education and area level income, and lower individual and area-level incomes were associated with lower gut  $\alpha$ -diversity, with a weaker association for education (10). Of note, the identified SES differences were only slightly attenuated with controls for diet, body mass index (BMI), and current health deficits, whereas the associations between these variables and the gut microbiome were significantly attenuated by adjustment for SES, suggesting that SES may be an important confounder that has not been previously accounted for in most microbiome–health research. These two papers are an important first step in describing associations of social factors and the microbiome, but data that can better test the mechanisms underlying these associations are needed. As we outline later in this review, socioeconomic conditions are key drivers of broader morbidity and mortality, and they also play an outsized role in patterning metabolic disorders, making socioeconomic factors an important confounder and/or effect measure modifier to consider in gut microbiome research.

## Race/Ethnicity and the Microbiome

Race/ethnicity reflects a range of influences on the microbiome, including common genetic ancestry, shared residence, culture, migration history, socioeconomic resources, and exposure to racism (35). Even with a small number of nonwhites in the HMP sample, investigators found that a “wide variety of taxa, gene families, and metabolic pathways were differentially distributed with subject ethnicity at every body habitat, representing the phenotype with the greatest number...of total associations with the microbiome” (61, p. 211). These incidental findings of strong associations with race/ethnicity suggest the need for further investigation in more diverse population-based studies, although these data are still limited. Data analyzed by Brooks et al. (11) from the American Gut Project (AGP) and the HMP identified 12 microbial genera and families that varied in abundance by ethnicity, and the investigators found that the associations of ethnicity with the microbiota were stronger in effect size than were associations of BMI, age, and sex. The AGP sample is a highly selected (26) volunteer sample; a very limited number of nonwhites (13 African Americans, 37 Hispanics, 88 Asian Pacific Islanders, and 1,237 whites) were included in the analysis, thus likely underestimating true differences in the gut microbiome by ethnicity. One interesting finding was that the typical association of *Christensenellaceae* with BMI was not consistent across ethnicities, suggesting the importance of sociodemographic factors in modifying microbiome–phenotype associations.

The Healthy Life in an Urban Setting (HELIUS) study, a population-based sample of Amsterdam residents with an oversample of ethnic minorities, is one of only a few studies to capture large numbers of respondents across different ethnic groups (439 Dutch, 367 Ghanaians, 280 Moroccans, 197 Turks, 443 African Surinamese, and 359 South Asian Surinamese). Ethnic Dutch respondents had the highest level of  $\alpha$ -diversity, whereas South Asian Surinamese had the smallest

(28). Ethnicity was also significantly associated with dissimilarities in gut microbiota composition as measured by the Bray–Curtis index, suggesting that individuals of the same ethnicity shared more similar microbiomes. Ethnicity was also associated with relative abundance of 559 out of 744 OTUs. In models adjusted for diet, age, sex, education, BMI, alcohol, smoking, physical activity, and area of residence, ethnicity remained the strongest predictor of both  $\alpha$ -diversity and  $\beta$ -diversity, while no other factor reached the effect size of ethnicity in the model; most associations of the other variables weakened or disappeared when adjusted for ethnicity. While the authors point to genetic factors underlying these associations, 94% of the non-Dutch participants were first-generation migrants, suggesting that early-life exposures prior to migration may have contributed.

## CASE STUDY: EPIDEMIOLOGY OF THE GUT MICROBIOME AND METABOLIC CONDITIONS

To better understand the general methodological challenges and implications of social contexts for microbiome research in epidemiology, we conducted a review of one focal area to assess the strengths and weaknesses of existing sample selection, research design, and inference. We first provide background on the association of the gut microbiome and metabolic disorders and then describe how the strong social patterning of metabolic conditions can serve as a model for thinking about social context and the gut microbiome. Next, we present findings from a mini-systematic review of existing literature on the gut microbiome and metabolic conditions, highlighting the types of samples used, the research design, adjustments for confounding, and use of compositionally robust measures of the microbiome. Implications for overall inference and future directions are discussed.

### The Gut Microbiome and Metabolic Conditions

The largest existing area of research linking the gut microbiome and health outcomes is that focused on the gut microbiome and obesity/metabolic traits. Studies on mice and humans have shown definitive links between the composition of the microbiota and obesity (7, 105). For example, transplanting fecal samples from obese mice to lean mice (7) and from twins discordant for obesity into germ-free mice (105) can successfully transmit adiposity phenotype. In turn, host gut microbiotas in humans change significantly after bariatric surgeries for weight loss, though one cannot parse out the separable influences of obesity and nutrition (31, 48). More generally, however, while the overall body of research finds connections between the gut microbiome and obesity, there is a lack of consensus about the size of effects and mechanisms underlying those relationships (13). Some specific results have failed to replicate across human studies, most notably the association of the *Firmicutes/Bacteroidetes* ratio, initially discovered in mice (6) and in early human studies (124) but not replicated in later, larger studies (34, 128, 133).

### SES, Race/Ethnicity, and Metabolic Conditions

Just as the gut microbiome is linked to obesity and metabolic disorders, so too are socioeconomic conditions and race/ethnicity. Obesity, for example, is strongly patterned by educational attainment and income, and these patterns originate in early life. Children whose parents have higher incomes, and especially higher educational attainment, are significantly less likely to become overweight and obese throughout adolescence (94). The socioeconomic gradient in obesity remains among adults. In the United States, the obesity rate for adult women living below 130% of the

poverty level is 45% for women compared with 30% for those living above 350% of the poverty level (95). Studies generally find that educational attainment is a more robust predictor of obesity than is income (17). While black men are less likely to be obese compared to white men, the reverse pattern is true for women (98). Other metabolic disorders, specifically hypertension, blood lipid levels, glucose levels, and insulin resistance, are also patterned by socioeconomic conditions (67). Moreover, the disparity emerges early; children whose parents had lower educational attainment had higher glucose levels and insulin resistance (125). Evidence indicates that these patterns are also stronger for education compared with income, specifically for higher levels of cholesterol and hypertension (130). Race differences are especially robust for glucose and hypertension—and while socioeconomic conditions explain some of these differences, they do not explain all of them; stress and discrimination pathways of significant interest are possible mediators (55).

In the context of microbiome–health research, research has shown that the health risks associated with obesity, such as diabetes and hypertension, vary by SES (12). For example, among those with similar BMI levels, those with lower educational attainment are at greater risk for both developing and dying from diabetes and cardiovascular disease—the possible explanations for which are linked to everything from occupational environments to variation in nutrition (43). A recent meta-analysis also found that obesity is a far greater risk for diabetes among those with low, compared with high, SES (127).

### Mini-Review: The Gut Microbiome and Metabolic Conditions

In this section, we explore the extent to which existing human studies on the gut microbiome and metabolic disorders employ standard epidemiological methodological practices and whether existing research on the gut microbiome and metabolic disorders accounts for the above social contextual factors. To this end, we conducted a mini-review of the current state of epidemiological research on the gut microbiome and metabolic conditions. We searched PubMed for human non-intervention studies analyzing associations between the fecal, colonic, or intestinal microbiome and metabolic phenotypes, including obesity, lipids, blood pressure, glucose, and metabolic syndrome, excluding major cardiovascular disease. We also mined references in existing systematic reviews in this area, passing relevant titles forward for abstract and full-text screening. We retained studies using untargeted 16S rRNA genomic sequencing survey methods, the most common measurement in recent studies. One author (A.R.) screened articles on the basis of abstracts and full text and extracted the following information from each article marked for inclusion:

1. Sample selection (recruitment). We identified samples as population representative or not representative on the basis of whether a form of random sampling was used. We defined community-based recruitment as involving home visit, phone or mail invitation, or in-person recruitment at a public school. Volunteer recruitment was defined as workplace, university, or existing clinical trial population. We defined samples relying on patients in a hospital or outpatient clinic as clinical samples.
2. Covariate control methods. These include adjustment [inclusion of covariate(s) in a regression model or propensity score, or analysis of residuals of the outcome variable regressed on covariate(s)], restriction [participants excluded in certain levels of covariate(s)], matching (controls selected to have similar levels of a covariate to cases), stratification (analysis conducted separately according to levels of a covariate), and Mendelian randomization. We recorded the covariate set separately for each method.
3. Study design. We classified studies as longitudinal cohort (explicitly used measurements from more than one time point in the analysis), cross-sectional (microbiome and phenotype measured at approximately the same time point and no selection based on disease of

interest), case control (microbiome and phenotype measured at approximately the same time point with disease/phenotype of interest used to select the sample), or unclear [could not be determined whether (*a*) different time points were involved or (*b*) sample selection depended on disease of interest]. Where interest was in the effect of the disease on the microbiome, what is typically classified as a case-control study was classified instead as cross-sectional (selection based on exposure).

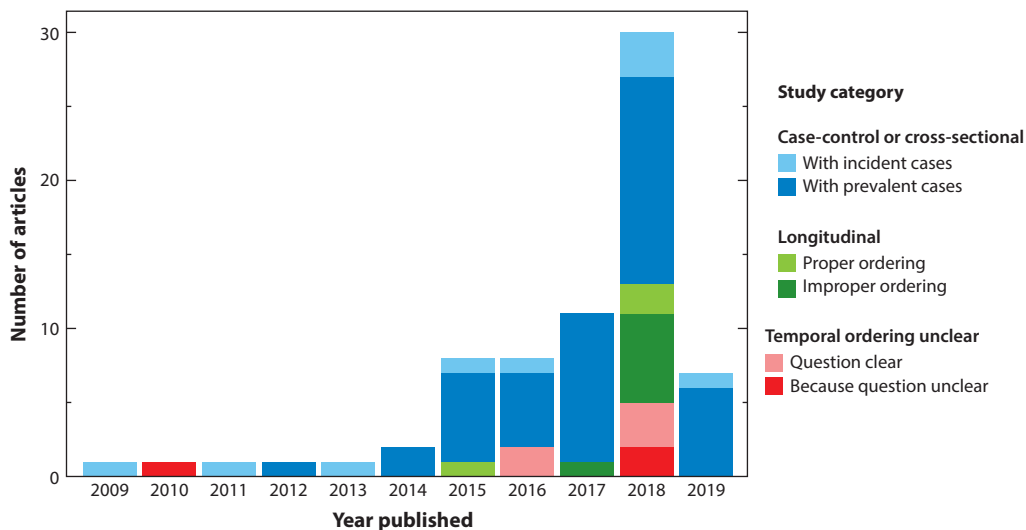
4. Temporal ordering. If interest was in the effects of the microbiome on disease, we required cross-sectional studies to measure recent incidence rather than prevalence of discrete disease in order to be classified as having proper temporal ordering and classified as improper all such studies measuring a continuous trait such as BMI or blood pressure. For longitudinal cohort studies, proper temporal ordering entailed microbiome measurement preceding diagnosis of a disease and, for continuous traits, at least one measurement following the microbiome. If interest was in the effect of the disease on the microbiome, cross-sectional or longitudinal studies with prevalent cases of disease were classified as proper.
5. Compositionally robust methods. A major validity issue that has often been overlooked in microbiome science has been the compositional structure of 16S data, in which each sample is a vector of counts representing the number of reads detected for each taxon, and typically only the relative proportions of each taxon within a sample are of interest. This type of data is referred to as a composition, meaning it carries only information about relative percentages of a total. In a composition, the act of normalizing to a total, as is commonly performed, is termed closure and creates spurious correlations between the elements, a feature noted by Karl Pearson in 1897 (100). These spurious correlations resulting from closure are a massive concern for microbiome science, resulting in roughly three-quarters of detected correlations between taxa being false and 60% of true correlations missed (36, 81). They also induce spurious correlations between the relative abundances and exposures or outcomes of interest (49). This problem likely also influences beta diversity findings, as common beta diversity metrics depend on relative abundances (42). Drawing on the work of Aitchison showing that only ratios between elements of a composition allow for the application of common statistical techniques (2), tools have recently been developed to analyze correlational taxon networks (36, 72), differential abundance (32, 84), and phylogenetic beta diversity (116) in a compositionally robust way.

For each study, we recorded which compositionally robust and nonrobust analytic methods were used. Nonrobust methods included analysis of differentially abundant (DA) taxa using raw counts or closed compositions, including direct comparisons (e.g., *t*-tests) of taxa percentages, as well as newer methods such as LEfSe (114), DESeq2 (80), and metagenomeSeq (98), which also rely on normalization methods not directly accounting for compositionality. Nonrobust methods also included rarefaction (equalizing the total number of reads across samples), beta diversity measures such as weighted and unweighted UniFrac (82) and Bray–Curtis distances (76), and correlational network methods using closure (e.g., Pearson’s correlation). Compositionally robust methods included DA using a log-ratio transform such as ANCOM (84) and ALDEx2 (32) or a log-linear model with a log-ratio-based offset (15), Aitchison (2) or PhILR (116) distance as beta diversity measures, and correlational network methods using log-ratio transforms such as SparCC (36) and SPEIC-EASI (72).

## Results

Of 1,899 studies meeting initial search criteria, 71 studies were selected on the basis of abstract and full-text review, 90% of which were published since 2015 (**Supplemental Table 1**). The majority





**Figure 1**

Study design and temporal ordering issues in the 16S microbiome literature on cardiometabolic phenotypes, 2009–2019 ( $n = 71$ ).

had small sample sizes (median = 100), although several published in the past 2 years have more than 1,000 participants (9, 50, 63, 75, 132, 133). Clinical and volunteer-based sampling dominated, with community-based recruitment less common ( $n = 13$ ). Among studies using community-based recruitment, only four used a sampling strategy aimed at population representativeness: In 2018, He et al. (50) used a multistage probability sample based in Guangdong, China; in 2017, Org et al. (96) used a random sample of a population register in Kuopio, Finland; in 2018, Rampelli et al. (104) aimed to recruit all children attending kindergarten and primary school in preselected municipalities across eight European countries; and in 2019, Sun et al. (120) used the CARDIA study, a representative sample of black and white adults in Minneapolis. Cross-sectional and case-control studies were the most common, but a substantial number of longitudinal cohort studies have recently been published, primarily in 2018 (**Figure 1**). Notably, in a surprising number of studies ( $n = 16$ ), it was impossible to determine the method of recruitment (23–25, 60, 66, 77, 102, 106, 112, 113, 118, 124, 135), and study design was unclear in  $n = 4$  [either because measurement time points were not specified (75) or selection based on outcomes was possible but unclear (79, 89, 124)].

The prevalence of studies over time by study design and temporal ordering categorizations is shown in **Figure 1**. In the majority ( $n = 39$ ) of studies, we classified temporal ordering as improper in some way. Most typically ( $n = 36$ ), these were case-control or cross-sectional studies that relied on prevalent phenotypes, and in nearly half ( $n = 7$ ) of studies in which temporal ordering was proper, the interest was in the effect of the phenotype on the microbiome, suggesting that the available data are more informative for this question. Longitudinal cohort studies achieved proper temporal ordering most frequently ( $n = 7$ ). Notably, temporal ordering was difficult or impossible to determine in 8 studies (see **Supplemental Table 1** for classification of specific studies).

**Supplemental Figure 1** catalogs the covariates controlled in some way by each study, according to the method used. Restriction and adjustment were used most frequently, although  $n = 7$  studies used no apparent method to control confounders. Nearly half ( $n = 32$ ) of studies excluded participants who had recently taken antibiotics/antimicrobials (1, 3, 23–25, 33, 34, 40, 47, 60, 65, 68, 73, 77–79, 86, 89–92, 97, 101–103, 106, 112, 120, 124, 126, 131, 136), and the majority ( $n = 42$ )

**Supplemental Material** >

restricted on the basis of health in some way (1, 3, 8, 14, 16, 23–25, 33, 34, 40, 44, 57, 62, 65, 66, 73, 77, 79, 86, 89–93, 96, 97, 101–103, 106, 112, 113, 120, 123, 126, 131, 134–136). Such studies typically excluded patients with major chronic diseases or cardiometabolic phenotypes (such as major cardiovascular disease) other than that being studied. Less than half ( $n = 34$ ) of studies used adjustment (1, 8, 9, 24, 25, 33, 38, 40, 46, 50, 59, 63, 64, 66, 68, 71, 75, 78, 79, 90, 91, 96, 97, 101, 104, 106, 118–120, 122, 123, 133, 136), typically for age and sex. Some studies ( $n = 8$ ) adjusted for diet in some way (9, 25, 33, 68, 71, 101, 120, 133). A few studies ( $n = 11$ ) used matching (27, 44, 70, 74, 75, 77, 78, 89, 103, 113, 124), typically for age and sex, and three studies (75, 78, 124) matched monozygotic twins, accounting for genetics and early-life environment. One study (132) used Mendelian randomization, which leverages genetic instrumental variables to adjust for measured and unmeasured confounders. Of note, only three studies adjusted for education (64, 119, 120), two for race/ethnicity (119, 120), and four for geographic location (23, 25, 50, 71).

Finally, we examined compositionally robust and nonrobust analytic methods used in each study. All 71 studies use at least one compositionally nonrobust method, and none used compositionally robust methods for differential abundance or beta diversity. In contrast, correlation network methods, which calculate correlations between taxa in order to determine groups of co-occurring taxa, were the only compositionally robust method observed in  $n = 5$  studies (23, 24, 40, 46, 106), whereas 7 used nonrobust correlation network methods (23, 24, 39, 96, 104, 122, 136).

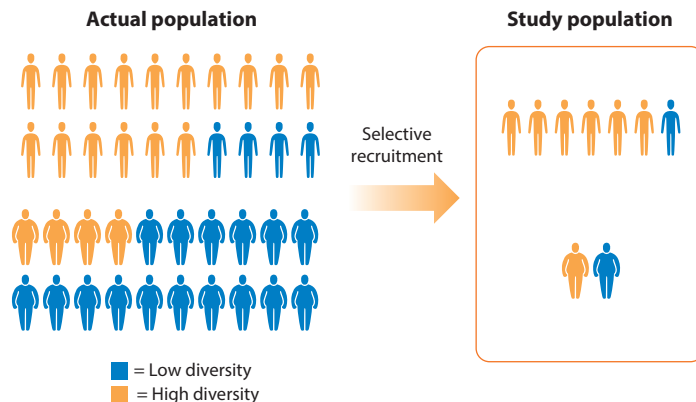
## Discussion

Over the past 5–10 years, there has been massive growth in both the number and the sample sizes of human observational studies examining associations between various metabolic phenotypes and the gut microbiome. Despite significant advances, our review highlights several methodological areas in need of innovation and attention, specifically issues around sample selection, study design, and confounding.

**Sample selection.** Only a small percentage of studies (4 of 71) had a population-based random sampling design, which is of great importance for the generalizability of findings. Twenty studies used volunteer recruitment, typically based in a workplace (56, 79, 133), a university (91), or existing clinical trials (5, 16, 44, 45, 57, 64, 93, 134). Several studies utilized data from the TwinsUK cohort (9, 46, 63, 75) recruited through media campaigns (88) and the American Gut project (8), in which participants were recruited online (85). Another 22 relied on in- or out-patient clinical samples (1, 3, 14, 18, 33, 38, 47, 58, 62, 65, 71, 73, 74, 89, 90, 101, 103, 119, 122, 123, 126, 131). Volunteer and convenience samples are known to be self-selected on both high SES and good health, with potentially serious implications for inference (37).

**Figure 2** provides a stylized illustration of how nonrandom samples selected for high SES can lead to an underestimate of associations between gut microbiome diversity and obesity. As previously detailed, the health risks associated with obesity are often found to be less severe for higher-SES individuals, likely reflecting more favorable underlying health profiles on both observable (smoking) and unobservable factors (early-life conditions, sleep, stress), all factors which likely affect the gut microbiome. Among the entire population of obese respondents, therefore, we would expect a higher fraction of high-SES individuals to have a “healthier,” or in this case more diverse, gut microbiome composition compared with lower-SES individuals. **Figure 2** shows how this selection would thus underestimate the association between diversity of the gut microbiome and obesity relative to the true association in the entire population by minimizing differences in the microbiome between obese and nonobese individuals. This type of selection bias may help explain the lack of reproducibility of results from animal models to human populations thus far





**Figure 2**

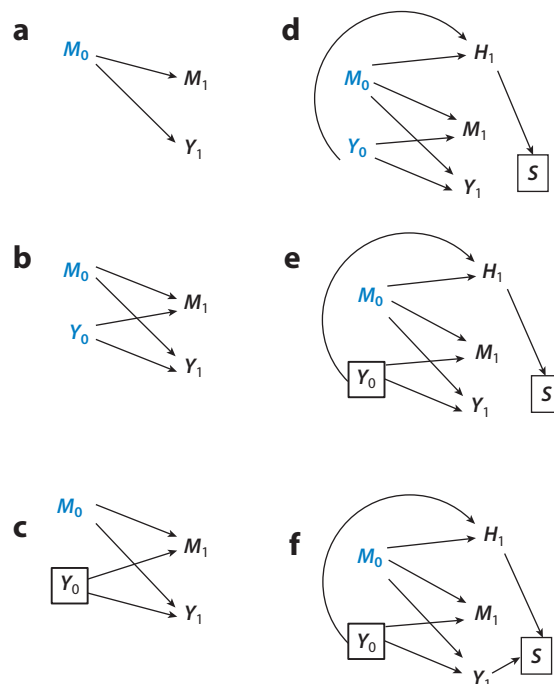
Implications of selection on socioeconomic status (SES) for obesity–microbiome associations. On the left, the actual population is 30% obese. Although the obese population is more likely to have low microbiome diversity, some fraction of obese, likely those of higher SES, will still have relatively high diversity levels. This selection bias in volunteer samples, which tend to be healthier and higher SES, can minimize differences between lean and obese individuals on diversity measures. In the actual population, while 80% of lean individuals and 20% of obese individuals will have high diversity, the select study population would be 85% and 50% respectively, thus underestimating the association between obesity and diversity.

and emphasizes the need for population-representative data sets with a full range of variability in SES (110), which is still very rare in this field.

**Confounder adjustment.** As in most observational research, causal inference on effects of the gut microbiota on host cardiometabolic phenotype is challenged by the fact that many exposures that impact the microbiome also affect health outcomes through different pathways. These include individual-level factors such as diet, smoking, physical activity, and prescription medications (especially antibiotics) (41); early-life factors such as birth mode and gestational age at birth (19); and broader population-level contexts such as geography, SES, and race/ethnicity (50). Studies included in our review typically aimed to account in some way for age, sex, antibiotics, and other medications, usually by restriction or regression adjustment (**Supplemental Figure 1**). Additionally, though nearly every study attempted to account for existing health status in some way, a possible bidirectional relationship between nearly all organ systems and the gut microbiome (115) makes systemic health one of the thorniest validity issues in this field. Thus, single-time-point studies conditioning on health status in any way risk creating collider bias, as illustrated in **Figure 3**. Longitudinal studies with proper temporal ordering are better situated to measure and adjust for such time-varying confounding (107).

One promising development is the use of Mendelian randomization (MR), employed in one study in our review (132). This technique capitalizes on findings from recent microbiome genome-wide association studies (GWAS) (129) to strengthen causal inference, potentially bypassing temporal ordering concerns, unmeasured confounders, and other complex forms of endogeneity present in host-microbiome interactions (121), under some fairly strong assumptions (20). Although its use is likely to increase in the coming years, MR in microbiome studies is currently challenging because of poor replicability in existing microbiome GWAS (129), limited functional understanding of observed genetic associations (21), and the need for very large samples (20) of which only a few currently exist owing to the expense of high-throughput sequencing. Despite the evidence reviewed above showing the strength of associations with race/ethnicity and SES

**Supplemental Material** >



**Figure 3**

Directed acyclic graphs illustrating common study design issues present in cardiometabolic microbiome human subjects literature. Nodes represent variables (*black*, measured; *blue*, unmeasured) and the arrows represent causal relationships. A square around a node means the analysis is conditional on that variable in some way, whether by adjusting for it, restricting on it, or through other means. Subscripts indicate time points. (a) A cross-sectional study where microbiome ( $M_1$ ) and disease outcome ( $Y_1$ ) are measured concurrently.  $M_1$  is unlikely to affect simultaneous disease ( $Y_1$ ) but is meant as a proxy for the previous microbiome,  $M_0$ . (b) A cross-sectional study where prevalent cases are analyzed.  $Y_1$  can now be a marker for previous disease,  $Y_0$ , which can affect  $M_1$ . (c) A cross-sectional study where incident cases are analyzed.  $Y_1$  is now no longer a marker for previous disease and confounding by  $Y_0$  is controlled. (d) A cross-sectional study where prevalent cases are analyzed and participants are selected ( $S$ ) according to health variables ( $H_1$ ). Selection bias exists owing to conditioning on a (descendent of a) collider,  $S$ . (e) A cross-sectional study where incident cases are analyzed and participants are selected ( $S$ ) according to health variables ( $H_1$ ). Selection bias in panel  $d$  is alleviated because a noncollider ( $Y_0$ ) on the collider path present in panel  $d$  is controlled. (f) A case-control study where incident cases are analyzed and participants are selected ( $S$ ) according to health variables ( $H_1$ ). Selection bias exists owing to conditioning on a collider,  $S$ .

and demonstrating that adjustment for these factors can reduce or eliminate the strength of focal microbiome–health associations, these factors were rarely considered.

**Study design and selection bias.** In **Figure 3**, we illustrate several issues concerning temporal ordering and selection in metabolic microbiome research using causal diagrams, where  $M_0$  and  $Y_0$  and  $M_1$  and  $Y_1$  represent the microbiome at baseline and follow-up, respectively (99). Many studies restrict eligibility to otherwise healthy individuals, illustrated in **Figure 3d**, where  $H_1$  represents health at baseline and  $S$  represents selection into the study. The rationale may be that health status can impact the microbiome and the disease of interest and is thus a confounder (59, 126). However,  $H_1$  could be affected by both prevalent metabolic syndrome ( $Y_0$ ) and previous microbiome ( $M_0$ ), so  $H_1$  is termed a collider, meaning a common effect of exposure and outcome (54, 99).

Restricting participation, or otherwise conditioning, on the basis of such a variable is known to induce selection bias, generating false associations that do not exist in the population (54). Heuristically, among otherwise healthy people, those who have metabolic syndrome are more likely to have a protective microbiome (54). Fortunately, we can control this bias by including only incident cases (i.e., controlling  $Y_0$ ) (**Figure 3e**). Alternatively, **Figure 3f** depicts a case-control study, where we would not be able to control selection bias by restricting to incident cases because, in a case-control study, selection has already occurred on the basis of case status (hence the arrow from  $Y_1$  to  $S$ ), and now  $S$  is a collider between  $M_0$  and  $Y_1$ , inducing selection bias. (For a more complete explanation, see Reference 57.)

Two major takeaways of **Figure 3** are (a) for both cross-sectional and case-control studies, restricting analysis to incident cases limits confounding by previous disease; and (b) restricting a case-control study to individuals without other health conditions (other than the disease in question) may result in selection bias that could be avoided by eliminating such exclusion criteria. Therefore, both unmeasured confounding and selection bias likely affect a huge swath of the literature on gut microbiomes and cardiometabolic phenotypes.

Longitudinal cohort studies on this topic are frequently more informative with regard to the causal question of whether the microbiome is involved in the etiology of disease. For example, in 2018, Rampelli et al. (104) conducted a longitudinal cohort study in which baseline fecal samples were collected for 70 children aged 2–9 years, all of whom were classified as normal weight on study entry and about half of whom developed overweight or obesity throughout the 4-year study period. Authors explored associations between baseline microbiome and weight change over the study period, controlling for age. Such a design eliminates the specific biases discussed above (a) because there is no selection based on health or other variables affected by both previous microbiome and previous disease (i.e., no collider bias), and (b) because all participants were normal weight at baseline, only incidence of overweight is observed. Additionally, baseline microbiome ( $M_0$  in **Figure 3**) has been observed rather than inferred from later microbiome ( $M_1$  in **Figure 3**), a much less risky strategy as gut microbiomes have been observed to change rapidly, for example in response to dietary changes (22). A nearly equivalent approach is the use of stored fecal samples in case-control studies, as in the study of incident type 1 diabetes conducted in 2015 by Kostic et al. (71).

**Analytic methods and compositional robustness.** Notably, none of the reviewed studies used compositionally robust techniques, with the exception of correlation networks, employed by five studies (23, 24, 40, 46, 106). The compositionally nonrobust methods used in most of the reviewed research call into question the truth of observed associations and suggest that findings from these studies are unlikely to replicate. However, this trend is expected, as the methods that have been most effectively disseminated and popularized, including UniFrac and Bray–Curtis distances and LEfSe and DESeq2, do not explicitly consider the compositional structure. Though developed in the 1980s, Aitchison’s work establishing the mathematical properties of compositions has only recently been considered in expert recommendations for microbiome data analysis (69), and compositionally robust differential abundance (32, 84) and phylogenetic distance (116) methods emerged several years later than other highly popular methods (82, 98, 114).

## CONCLUSION

Methodologically, as we move forward from the early days of microbiome research, it is clearly time for epidemiology to work toward best practices such as using compositionally robust measures and attending properly to such basic issues as temporal order, avoidance of selection bias, and

adjustment for confounding. In an alarming number of the studies we reviewed (**Supplemental Table 1**), the sample recruitment and study design could not be ascertained from the paper's methods, a serious problem that we can surely overcome collectively in our own writing and reviewing in this emerging field.

Overall, descriptive epidemiology of the microbiome by social context remains limited, especially with the current lack of population-representative, randomly selected samples. Thus far, it is clear that variables measuring important aspects of the social environment—race/ethnicity and SES—show strong associations with the gut microbiome, often explaining more variation than most other salient individual-level determinants such as diet. Given the strong social patterning of obesity and metabolic conditions, existing studies on the gut microbiome and metabolic conditions that do not account for SES and race/ethnicity are at strong risk of serious bias. At the same time, the strong associations between social context, the gut microbiome, and metabolic conditions across the life course provide an opportunity to explore the gut microbiome as a mechanism underlying social disparities in metabolic disease.

Moreover, microbiome–health associations themselves are increasingly found to vary across these social contexts, compelling researchers to carefully consider the inferences they can make from homogeneous and volunteer samples. A notable 2018 paper highlighted the importance of geography in the generalizability of microbiome–disease associations using data from 14 districts within one large province in China (51). The effect sizes of geographic variation dominated in predicting gut microbiome composition, exceeding those of metabolic diseases, type 2 diabetes, obesity, and fatty liver. The authors applied a disease model for type 2 diabetes trained in one location to another location and found predictive power was reduced to no better than random guessing, suggesting that “healthy” reference baselines for gut microbiota can be heavily dependent on location. Taken together with similar results for SES and race/ethnicity, this result strongly suggests that predictive models will need to consider social and geographic contexts seriously before these models can be broadly applied and that this population-level perspective may be key to ultimately understanding and intervening on the microbiome. As Geoffrey Rose might say, Why do some individuals have sick microbiomes? is a different question from, Why do some populations have sick microbiomes?

## DISCLOSURE STATEMENT

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