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Sick individuals and sick (microbial) populations: Challenges in epidemiology and the microbiome

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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 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 non-compositionally robust measures, and a lack of attention to social factors as confounders or effect modifiers.

Keywords

microbiome; microbiota; SES; race/ethnicity; social; metabolic; epidemiology

I. INTRODUCTION

Since the first publication of results from the Human Microbiome Project (HMP) in 2012 (60), research on the microbiome- the trillions of microorganisms that inhabit the human body and their genes-has grown exponentially[1], generating new knowledge on everything from how diet affects the microbiome to how the microbiome may influence the brain, behavior, and mental illness (4; 116). Robust evidence points to a stronger role for the "environment" in shaping the human gut microbiome relative to genetics (110), compelling researchers to better define and measure the "environment" to understand the

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[1]From 43 papers in 2000 to 5032 in 2018, via PubMed search for "microbiota OR microbiome AND human," 5/24/19.

role of the microbiome in human health and disease (52). 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 towards actionable knowledge (82). Thus while it remains important to understand microbiome function at the molecular level with an eye towards novel prognostic and treatment breakthroughs, it is equally important to "zoom out" and consider a population perspective in microbiome research (108). 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 (108). Social and geographical context, for instance, are emerging as crucial determinants of both the microbiome itself as well as modifiers of existing microbiome-health associations (28; 49). Epidemiology is well-poised to make important contributions to the description of the microbiome across person, place, and time, as well as to improving population-based research design and causal inference to understand how the microbiome impacts health and disease across the life course (107).

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 forward towards actionable knowledge. While 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(60). 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 socioeconomic status (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 (29; 52). We define socioeconomic status (SES) as reflecting human capital and economic resources such as education, income and wealth, and occupation. Socioeconomic resources can also be conceptualized and measured at the neighborhood level. Though 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 (51).

To date, we identified two studies that specifically link varying socioeconomic conditions to alterations in the gut microbiome (10; 86). Miller, et al. related a composite indicator of neighborhood socioeconomic status to the microbiota composition of 44 healthy volunteers in Chicago, finding higher α-diversity and greater abundance of *Bacteroides* with increased neighborhood socioeconomic status (86). In a large sample of UK Twins, Bowyer, et al identified associations between individual and area-level income and diversity and relative abundance of OTUs in the gut microbiome (10). Community composition measured by Bray-Curtis dissimilarity was found to differ by education and area level income, and lower individual and area-level income was associated with lower gut α-diversity, with a weaker association for education (10). Of note, the identified SES differences were only slightly attenuated with controls for diet, BMI, and current health deficits, while 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, not only are socioeconomic conditions a key driver of broader morbidity and mortality, they 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 in this area.

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 (34). Even with a small number of non-whites 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 (60)." 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. Brooks et al. analyzed data from the American Gut Project (AGP) and the HMP and identified 12 microbial genera and families varied in abundance by ethnicity and that the associations of ethnicity with the microbiota were stronger in effect size than BMI, age, and sex (11). It should be noted that the AGP sample is a highly selected (25) volunteer sample with a very limited number of non-whites (13 African-Americans, 37 Hispanics, 88 Asian Pacific Islanders, and 1237 Caucasians) 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 residents of Amsterdam with an oversample of ethnic minorities, is one of few studies to capture large numbers of respondents across different ethnic groups (439 Dutch, 367 Ghanians, 280 Moroccons, 197 Turks, 443 African Surinamese and 359 South-Asian

Surinamese). Ethnic Dutch respondents had the highest level of α-diversity, with South-Asian Surinamese having the smallest (27). Ethnicity was also significantly associated with dissimilarities gut microbiota composition as measured by 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. Intriguingly, in models adjusted for diet, age, sex, education, BMI, alcohol, smoking, physical activity, and area of residence, ethnicity remained the strongest predictor of both α-diversity and β-diversity, while no other factor reached the effect size of ethnicity in the model, and 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.

II. Case Study: Epidemiology of the gut microbiome and metabolic conditions

In order 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, 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; 104). For example, transplanting fecal samples from obese mice to lean mice (7) and from twins discordant for obesity into germ-free mice (104) 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 (30; 47). More generally, however, while the overall body of research finds connections between the gut microbiome and obesity, there is a lack of consensus as to the size 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 (33; 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 through adolescence (93). The socioeconomic gradient in obesity remains among adults. In the U.S., the obesity rate for adult women living below 130 percent of the poverty level is 45 percent for women compared to 30 percent for those living above 350 percent of the poverty level (94). Studies generally find that educational attainment is a more robust predictor of obesity than is income (17). While white men are less likely to be obese compared to white men, the reverse pattern is true for women (97). Other metabolic disorders, specifically hypertension, blood lipid levels, glucose levels and insulin resistance are also patterned by socioeconomic conditions (66). Moreover, the disparity emerges early; children whose parents had lower educational attainment had higher glucose levels and insulin resistance (125). There is evidence that these patterns are also stronger for education compared to 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 this difference, they do not explain all of these differences, with stress and discrimination pathways of significant interest as possible mediators(54).

Importantly in the context of microbiome-health research, it has been shown that the health risks associated with obesity, such as diabetes and hypertension, vary by socioeconomic status (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 (42). A recent meta-analysis also found that obesity is a far greater risk for diabetes among those with low, compared to high, socioeconomic status (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 of 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 (AR) screened based on the abstract 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 based on 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 students, or an existing clinical trial population. We defined samples relying on patients in a hospital or outpatient clinic as clinical samples.

(2) Covariate control methods: 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 effect 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 precededing 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*.

(6) Compositionally robust methods: A major validity issue that has been often 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 (99). These spurious correlations resulting from closure are a massive concern for microbiome science, resulting in roughly ¾ of detected correlations between taxa being false, and 60% of true correlations missed (35; 80). Importantly, they also induce spurious correlations between the relative abundances and exposures or outcomes of interest.(48) This problem likely also influences beta diversity findings, as common beta diversity metrics depend on relative abundances (41). 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 analyze correlational taxon networks (35; 71), differential abundance (31; 83), and phylogenetic beta diversity (115) in a compositionally-robust way.

For each study we recorded which compositionally robust and non-robust analytic methods were used. Non-robust methods included analysis of differentially abundance (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 (113), DESeq2 (79) and metagenomeSeq (97), which also rely on normalization methods not directly accounting for compositionality. Non-robust methods also included rarefaction (equalizing the total number of reads across samples), beta diversity measures such as weighted and unweighted UniFrac (81) and Bray-Curtis distances (75), and correlational network methods using closure (e.g. Pearson's correlation). Compositionally-robust methods included DA using a log-ratio transform such as ANCOM (83) and ALDEx2 (31) or a log-linear model with a log-ratio-based offset (15), Aitchison (2) or PhiLR (115) distance as beta diversity measures, and correlational network methods using log-ratio transforms such as SparCC (35) and SPEIC-EASI (71).

Results

Of 1899 studies meeting initial search criteria, 71 studies were selected based on abstract and full-text review, 90% of which were published since 2015 (Supplemental Table 1). The majority had small sample sizes (median=100), although several published in the past 2 years have over 1000 participants (9; 49; 62; 74; 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: He et al. (2018) used a multistage probability sample based in Guangdong, China (49); Org et al. (2017) used a random sample of a population register in Kuopio, Finland (95); Rampelli et al. (2018) aimed to recruit all children attending kindergarten and primary school in pre-selected municipalities across eight European countries (103); and Sun et al. (2019) used the CARDIA study, a representative sample of black and white adults in Minneapolis (120). Cross-sectional and case control studies were the most common, but a substantial number of longitudinal cohort studies have recently been published, primarily in the last year (Figure 1). Notably, in a surprising number of studies (n=16) it was impossible to determine the method of recruitment (22-24; 59; 65; 76; 101; 105; 111; 112; 118; 124; 135), study design was unclear in n=4 (either because measurement time points were not specified (74) or selection based on outcomes was possible but unclear (78; 88; 124)).

The prevalence of studies over time by study design and temporal ordering categorizations are 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 phenotype on the microbiome, suggesting this is a question for which the available data are more informative. 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 catalogues 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 recently on antibiotics/antimicrobials (1; 3; 22–24; 32; 33; 39; 46; 59; 64; 67; 72; 76–78; 85; 88–91; 96; 100–102; 105; 111; 120; 124; 126; 131; 136) , and the majority (n=42) restricted on health in some way (1; 3; 8; 14; 16; 22–24; 32; 33; 39; 44; 56; 61; 64; 65; 72; 76; 78; 85; 88–92; 95; 96; 100–102; 105; 111; 112; 120; 123; 126; 131; 134–136). Such studies typically excluded patients with major chronic diseases or cardiometabolic phenotypes (such as major CVD) other than that being studied. Less than half (33) of studies used adjustment (1; 8; 9; 23; 24; 32; 37; 39; 45; 49; 58; 62; 63; 65; 67; 70; 74; 77; 78; 89; 90; 95; 96; 100; 103; 105; 118–120; 122; 123; 133; 136), typically for age and sex. Some studies (n=8) adjusted for diet in some way (9; 24; 32; 67; 70; 100; 120; 133). A few studies (n=11) used matching (26; 44; 69; 73; 74; 76; 77; 88; 102; 112; 124), typically for age and sex, and three studies (74; 77; 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 (63; 119; 120), two for race/ethnicity (119; 120), and four for geographic location (22; 24; 49; 70).

Finally, we examined compositionally robust and non-robust analytic methods used in each study. All 71 studies use at least one compositionally non-robust 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 (22; 23; 39; 45; 105), whereas 7 used non-robust correlation network methods (22; 23; 38; 95; 103; 122; 136).

Discussion

Over the past 5-10 years, there has been massive growth in both the number and 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.

Of great importance for the generalizability of findings, only a small percentage of studies (4 of 71) had a population-based random sampling design. 20 studies used volunteer recruitment, typically based in a workplace (55; 78; 133), university (90), or existing clinical trials (5; 16; 43; 44; 56; 63; 92; 134). Several studies utilized data from the TwinsUK cohort (9; 45; 62; 74) recruited through media campaigns (87), and the American Gut project (8), in which participants were recruited online (84). Another 22 relied on in- or out-patient clinical samples (1; 3; 14; 18; 32; 37; 46; 57; 61; 64; 70; 72; 73; 88; 89; 100; 102; 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 (36).

Figure 2 provides a stylized illustration of how non-random samples selected for high SES sample 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 a 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 to 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 non-obese individuals. This may help explain lack of reproducibility of results from animal models to human populations thus far and emphasizes the need for population representative datasets with a full range of variability in SES (109), 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) (40), early life factors such as birth mode and gestational age at birth (19), as well as broader population-level context such as geography, SES, and race/ethnicity (49). Studies included in our review typically aimed to account in some way for age, sex, antibiotics, 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, possible bidirectional relationship between nearly all organ systems and the gut microbiome (114) makes systemic health one of the thorniest validity issues in this field. This means that 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 (106).

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 (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 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 (117), 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 and that adjustment for these factors can reduce or eliminate the strength of focal microbiome-health associations, these 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. (98). Many studies restrict eligibility to otherwise healthy individuals, illustrated in figure Supplemental Figure 1D, 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 (58; 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 (53; 98). Restricting participation, or otherwise conditioning, based on such a variable is known to induce selection bias, generating false associations that do not exist in the population (53). Heuristically, among otherwise healthy people, those who have metabolic syndrome are more likely to have a protective microbiome (53). Fortunately, we can control this bias by including only incident cases (i.e., controlling Y_0) (Supplemental Figure 1E). Alternatively, Supplemental Figure 1F depicts a case-control study, where we would not be able to control selection bias by restricting to incident cases. This is because, in a case-control study, selection has already occurred based on 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 56.)

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 as to the causal question of whether the microbiome is involved in the etiology of disease. For example, Rampelli et al. (2018) 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 because (a) 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) since 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 (21). A nearly equivalent approach is the use of stored fecal samples in case-control studies, as in the study of incident T1D conducted by Kostic et al. (2015) (70).

Analytic methods and compositional robustness.

Notably, none of the reviewed studies used compositionally-robust techniques, with the exception of correlation networks, employed by 5 studies (22; 23; 39; 45; 105). The compositionally non-robust methods used in the majority 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, 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 (68), and compositionally-robust differential abundance (31; 83), and phylogenetic distance (115) methods emerged several years later than other highly popular methods (81; 97; 113).

III. Conclusion

Methodologically, as we move forward from the early days of microbiome research, it is clearly time for epidemiology to work towards best practices such as the use of compositionally robust measures and as proper attention to basic issues as temporal order, avoiding 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 which we can surely collectively overcome 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 socioeconomic status- 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 take account for SES and race/ethnicity are at strong risk serious bias. At the same time, the strong associations between social context, the gut microbiome, and metabolic conditions across the life course provides 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 homogenous and volunteer samples. A recent notable paper highlighted the importance of geography in the generalizability of microbiome-disease associations using data from 14 districts within one large province in China (50). 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 strongly suggests that predictive models will need to seriously consider social and geographic context before they 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?’

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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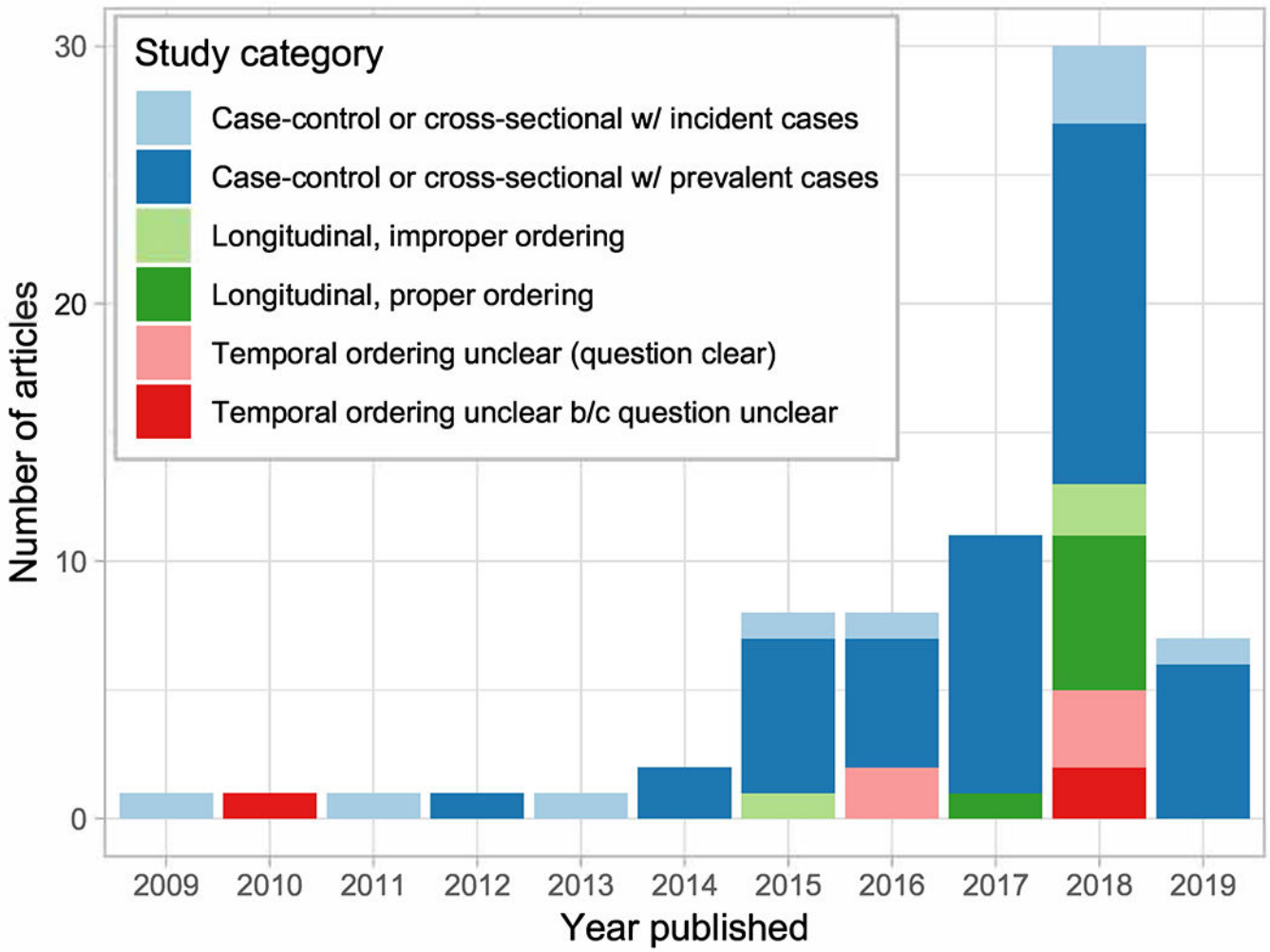

Figure 1.
Study design and temporal ordering issues in the 16S microbiome literature on cardiometabolic phenotypes, 2009-2019 (n=71).

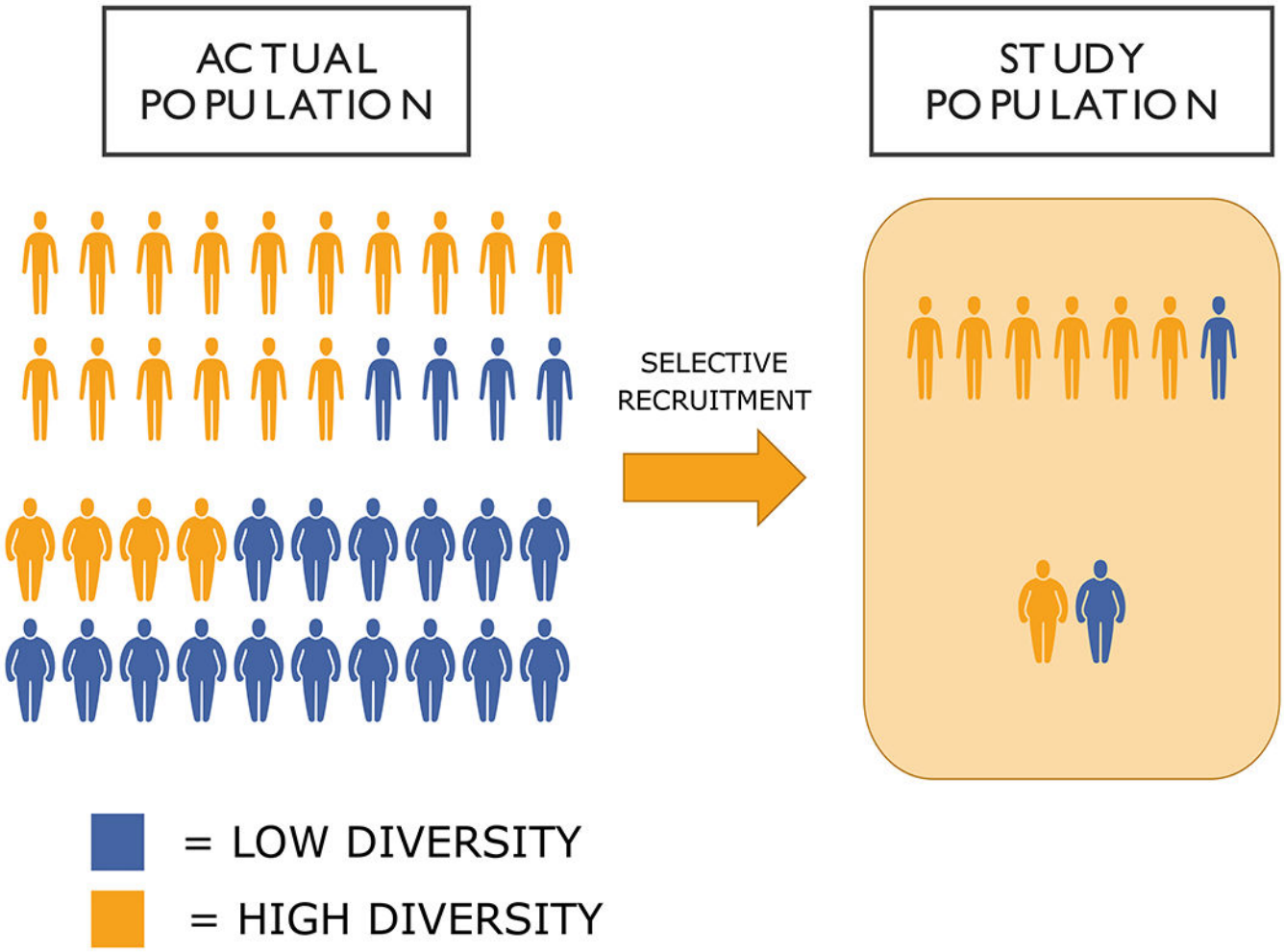

Figure 2:
Implications of selection on SES for obesity-microbiome associations. On the left, the actual population is 30% obese. While the obese population is more likely to have low microbiome diversity, some fraction of obese, likely those of higher SES, will still will have relatively high diversity levels. In a self-selected study sample, which tends to be healthier and higher SES, this 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.

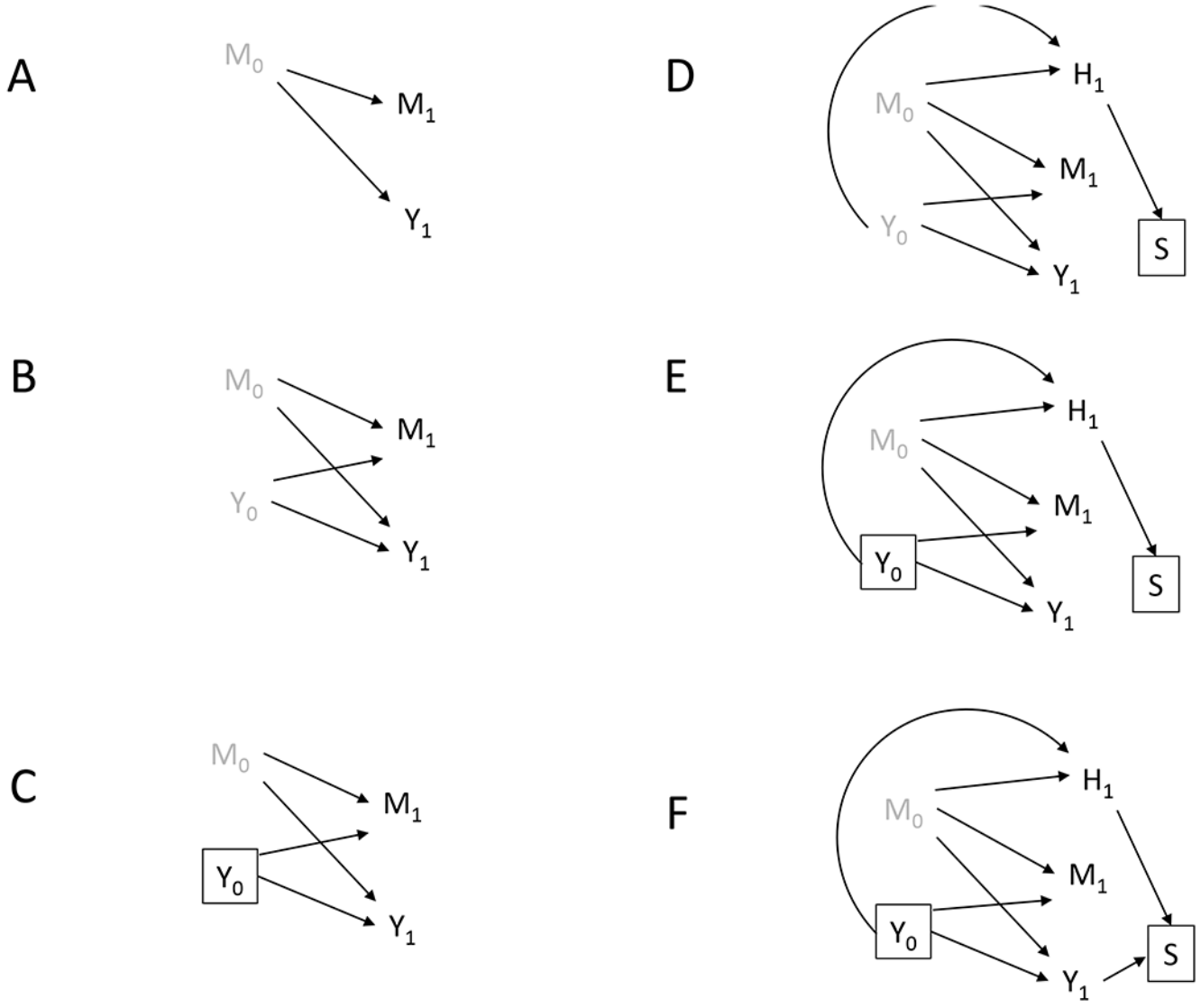

Figure 3.

Directed acyclic graphs illustrating common study design issues present in cardiometabolic microbiome human subjects literature. Nodes represent variables (black=measured, grey=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 other means. Subscripts indicate time points. (A) A cross-sectional study where microbiome (M_1) and disease outcome (Y_1) are measured concurrenty. M_1 is unlikely to affect simulatenous disease (Y_1), but is meant as a proxy for 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 anlyzed. 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 due 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 (D) is alleviated because a non-collider (Y_0) on the collider path present in (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 due to conditioning on a collider, S.