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INVITED REVIEW

Conducting research on diet–microbiome interactions: A review of current challenges, essential methodological principles, and recommendations for best practice in study design

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Abstract

Diet is one of the strongest modulators of the gut microbiome. However, the complexity of the interactions between diet and the microbial community emphasises the need for a robust study design and continued methodological development. This review aims to summarise considerations for conducting high-quality diet–microbiome research, outline key challenges unique to the field, and provide advice for addressing these in a practical manner useful to dietitians, microbiologists, gastroenterologists and other diet–microbiome researchers. Searches of databases and references from relevant articles were conducted using the primary search terms 'diet', 'diet intervention', 'dietary analysis', 'microbiome' and 'microbiota', alone or in combination. Publications were considered relevant if they addressed methods for diet and/or microbiome research, or were a human study relevant to diet–microbiome interactions. Best-practice design in diet–microbiome research requires appropriate consideration of the study population and careful choice of trial design and data collection methodology. Ongoing challenges include the collection of dietary data that accurately reflects intake at a timescale relevant to microbial community structure and metabolism, measurement of nutrients in foods pertinent to microbes, improving ability to measure and understand microbial metabolic and functional properties, adequately powering studies, and the considered analysis of multivariate compositional datasets. Collaboration across the disciplines of nutrition science and microbiology is crucial for high-quality diet–microbiome research. Improvements in our understanding of the interaction between nutrient intake and microbial metabolism, as well as continued methodological innovation, will facilitate development of effective evidence-based personalised dietary treatments.

KEYWORDS
nutrition, microbiota, gastrointestinal tract

INTRODUCTION

Diet has long been recognised as a significant modulator of human health. It is now clear that the gastrointestinal (GI) microbiota is an essential mediator of this influence of diet on health. Dietary substrates are a key determinant of the structure and function of the GI microbiome, influencing the production of metabolites and microbe–host interactions. Key observational studies[1–3] and short-term extreme diet intervention trials[1,4] provide examples of the nature and extent of its effect in the long- and short-term, respectively. For example, fibre deprivation alongside markedly increased

fat and protein intake has notable disruptive effects on microbial community structure, including the abundance of plant-metabolising Firmicutes.[4] As such, the microbiome has become an attractive target for dietary interventions aiming to modulate health outcomes, with particular relevance for conditions where the microbiome is considered important in disease pathogenesis (e.g., obesity, inflammatory bowel disease and irritable bowel syndrome).[5–7]

There are intrinsic complexities with respect to conducting studies in humans to assess diet–microbiome relationships. For example, humans are both free to select the foods that they consume and are relied upon for an accurate self-report of dietary intake. This leads to significant challenges, with under-reporting estimated to occur in 18–54% of participants in large population surveys.[8] Individuals also consume a wide variety of foods and, to date, food composition databases inadequately capture all dietary compounds that may have relevant effects on the microbiota. Furthermore, although the advent of high throughput sequencing technologies means that it is now possible to profile the majority of the microbiota in a rapid manner, challenges remain in linking these data to dietary intake and health outcomes, and finding consensus across studies. This may partly be a result of the significant inter-individual variation in the composition of the microbial community in the human gut, which may prime certain individuals for response/non-response to dietary interventions.[9]

To address these challenges in human diet–microbiome research, partnership across the disciplines of nutrition science and microbiology for planning, design, implementation, data analysis and interpretation is needed. The aim of this

TABLE 1 Dietary assessment methods and the key advantages and disadvantages that are relevant for diet–microbiome research

Dietary assessment method	Description	Output
Weighed food record	Participants weigh and record all food and beverages in real-time Usual duration 3–7 days	Energy, fibre, macronutrients, micronutrients, additional food constituents (depending on food composition data) Food groups Number of meals
Unweighed food record	Participants record estimated quantities of food and beverages in real-time Usual duration 3–7 days	As for weighed food record
24-h recall *Examples of online versions*: ASA24, myfood24, Intake24	Food and fluid intake between midnight to midnight day prior collected through structured interview with trained interviewer	As for weighed food record
Food frequency questionnaire *Examples:* Harvard FFQ, EPIC FFQ, AES, Food4Me	Questionnaire that assesses frequency of consumption of individual foods over a defined period (e.g., 1 year) Most include 80–120 items	Energy, fibre, macronutrients, micronutrients and additional food constituents (dependent on food composition data) Food groups
Diet quality and dietary patterns		
Diet quality *Examples:* HDI, HDS, HEI, AHEI, MDS	*A priori* score measuring overall healthfulness of the diet based on current evidence	Components aggregated to obtain a final score. Higher score indicates better diet quality
Diet pattern analysis	*A posteriori* approach Derives patterns using principal components/ exploratory factor analysis or cluster analysis. Patterns such as 'prudent' or 'Western' can be derived	Identifies foods consumed together (principal components analysis) or clusters individuals with differing dietary intakes (cluster analysis)

Abbreviations: AES, Australian Eating Survey; AHEI, Alternate Healthy Eating Index; ASA-24, Automated Self-administered 24-hour Dietary Assessment Tool; FFQ, food frequency questionnaire; HDI, Healthy Diet Indicator; HDS, Healthy Diet Score; HEI, Healthy Eating Index; MDS, Mediterranean Diet Score.

review is to discuss ongoing challenges in diet–microbiome research and issues for best-practice study design, as the field moves towards a goal of personalised nutrition for disease prevention, as well as therapeutic diets for a broad spectrum of microbiome-associated disorders.

Ongoing challenges in diet–microbiome research

Precision in dietary data collection

One of the most acknowledged intrinsic complexities of dietary research is that dietary intake data are measured subjectively via self-report. Provision of clear instructions to participants prior to completion of diet recording, utilisation of resources (e.g., food models) to enhance portion size accuracy, and comprehensive cross-checking of collected data are simple yet often overlooked strategies that can improve accuracy of dietary data. With regard to energy intake, implausible data can be identified using calculations based on low and high energy intake cut-offs[10] or predicted energy requirements.[11] The expertise of a nutrition professional for implementing these measures, as well as coding and analysing dietary data, is essential. There has also been increasing interest in capturing 24-h recall through mobile phone-based applications, some of which employ image-based technology. These tools are potentially of great value, particularly for reducing respondent burden and improving adherence and accuracy.[12] Table 1 details the major dietary

Study design	Advantages in diet–microbiome research	Disadvantages in diet–microbiome research
Experimental 'whole diet' interventions; supplementation studies to check background intake Cohort Case–control Cross-sectional Longitudinal	Most precise measure of actual dietary intake Useful for associating very recent dietary intake with microbiota profile Good agreement with biological dietary biomarkers	Very burdensome for participant Burdensome for researcher May influence eating behaviour May require assessment of inter-observer agreement between coders
As for weighed food record	Less burdensome for participant than weighed food record Useful for associating very recent dietary intake with microbiota profile	Burdensome for researcher May influence eating behaviour Participants may under-or over-estimate quantities
Cohort Case–control Cross-sectional Longitudinal	Low burden for participant Online versions available Multiple 24-h recalls demonstrate good agreement with dietary biomarkers Single 24-h recall acceptable for large cross-sectional studies	Risk of recall error Requires trained interviewer Interviewer bias (data accuracy dependent on interviewer expertise, consistency between interviewers) Single 24-h recall usually not appropriate due to day-to-day variation in dietary intake
Experimental studies requiring long-term diet data Cohort Case–control Cross-sectional Longitudinal	Accounts for weekly/seasonal variation in intake Useful for assessment of habitual diet–microbiome associations Low burden for participant Simple to administer Practical for large scale studies Validated tools available for specific populations, specific nutrients	Time consuming for participant (up to 60 min) Requires mathematical skill to calculate intake using frequency categories Infrequently consumed foods may be missed due to fixed food lists Greater risk of under-reporting and error compared with other methods
Cohort Case–control Cross-sectional Longitudinal	Accounts for complexity of the diet and interactive effects of dietary factors Many indices validated by relating index score against health outcomes	Majority require nutrient intake assessment (i.e. food record, 24-h recall or FFQ) for calculating final score
Cohort Case–control Cross-sectional Longitudinal	Accounts for complexity of the diet and interactive effects of dietary factors Can be used as a covariate to determine if the effect of a nutrient is independent of the overall dietary pattern	Requires nutrient intake assessment Patterns empirically derived from data not from diet-health evidence Arbitrary decisions required (e.g., food groups, number of factors/clusters to be retained)

assessment methods and their key advantages and disadvantages in diet–microbiome research.

To overcome the limitations of self-report, biological markers can also be used as surrogate markers of recent dietary intake. Urinary nitrogen, as a marker of protein intake, is the most well validated of these biomarkers,[13] although a variety of other metabolites have also been used to estimate food intake, including wholegrain wheat or olive oil, or nutrients, such as carbohydrate and vitamins.[14] Recently, metabarcoding techniques have also been used to quantify the plant component of humans diets that is recovered in stool samples.[15] Currently, these biomarker methods are limited to a finite list of dietary constituents, can be expensive, and the validity, reproducibility and sensitivity of some available biomarkers is still suboptimal.[16] Furthermore, some metabolite biomarkers may also be a by-product of microbial activity, which complicates their use for estimating dietary intake. Although diet self-report will continue to be necessary for years to come, future research aiming to refine existing biomarkers and identify new biomarkers of food intake using cost-effective technologies will be important for advancing our understanding of diet–microbiome relationships.

Measuring dietary substrates available to the microbiome

Food composition data

Despite ongoing efforts to more precisely quantify dietary intake, challenges remain with regard to applying this to diet–microbiome research. First, although many dietary constituents relevant to diet–microbe interactions have been measured in foods, not all are readily quantifiable in human diets or have simply not yet been measured comprehensively across the entire food supply. For example, total fibre intake can be estimated using current methods of dietary analysis, although this is without discrimination of fibre types. This is an important shortcoming considering the vastly different physico-chemical attributes of individual fibres (including degree of polymerisation, viscosity), which influences their availability for microbial fermentation.[17] Similarly, plant phenols have been shown to alter the composition of the human GI microbiota,[18] although they are rarely included in food composition databases. Progress in composition analysis of these food components and development of validated tools to measure their intake will be an important step forward. Even with such advances, measurement of availability of dietary components to intestinal microbes will remain problematic. Food composition tables are based on chemical analysis of foods, which fails to account for variation in bioavailability of dietary substrates, particularly those found in plant foods.[17] This, together with inter-individual variation in absorption, especially of minerals,[19] limits our ability to precisely quantify the level of dietary substrates accessible to microbes.

Dietary assessment: Granular or global?

Another relevant consideration is whether a nutrient-centric or global dietary assessment should be conducted. Much of the cross-sectional work examining diet–microbiome associations in health[3] or disease[20–22] has focussed on the relationship between individual nutrients and the microbiome. This has substantially enhanced our understanding of these relationships and will continue to be relevant to measure. However, data at this granular level are increasingly recognised as failing to capture the complexity of the diet–health relationship. Dietary intake occurs in the form of food and meals, and assessing diet in this way may better explain the impact of diet on the microbiome.[23,24] As such, a global dietary parameter, such as diet quality,[25,26] or dietary pattern analysis,[27] may be an important adjunct measure. These may prove invaluable as they can be used to capture the combination and interaction of foods and nutrients consumed and assess the overall healthfulness of the diet relative to healthy eating guidelines or adherence to a specific beneficial dietary pattern such as the Mediterranean diet. Complex modelling of food intake using a tree-based alpha-diversity measure of food diversity is also possible. A recent study utilised this novel method to show that food choices are very variable across a 17-day period and that foods themselves are more strongly associated with the microbiome profile of healthy individuals than macronutrient intake, which remains relatively stable.[9] These data further support the notion that traditional analysis of macronutrients alone may fail to uncover diet–microbiome relationships.

Relevance of microbial niches

As a result of digestion and absorption, nutrients are not universally available to microbes across the various niches of the human GI tract. In combination with variable digestive processes and motility, this leads to a unique microbiome across each GI region.[28,29] Interpreting links between dietary intake and the GI microbial community must therefore consider substrate availability in the region sampled. However, to date, much of our understanding of diet–microbiome interactions is based on microbial profiles of stool, as a result of the relative ease of collection. Here, the luminal microbial compartment of the distal colon is reflected,[30] which represents the bulk of GI microbial biomass. Dietary components that are host-indigestible, namely fibre, form the key substrates for colonic fermentation, and are therefore central to microbial composition in this region. However, spill-over of excess dietary protein and fat,[31,32] as well as the other impacts of macronutrients on host physiology, such as bile production,[33] can also influence colonic microbes, and this must be considered particularly in the interpretation of whole diet interventions in which multiple dietary changes are required. By contrast, the small intestinal microbiome is comparatively poorly characterised as a result of lower microbial densities and difficulties in sampling. However, emerging evidence suggests it also plays an important role in disease pathophysiology,[34,35] and is involved

in immune-microbe interactions and modulating small intestinal permeability.[36] Although the majority of digestible dietary substrates are absorbed here, including sugars, lipids and amino acids, microbes are in competition with the host for these simple substrates, signifying the important modulatory role of diet in this region.[37]

Microbes adherent to the mucosa, although lower in biomass, have a greater ability to interact directly with host cells compared with luminal microbes.[38–40] Substantial evidence supports these compartments as distinct communities.[28,40–42] Enriched with mucin-utilising organisms, the mucosal compartment, particularly in the colon, has been shown to play important regulatory roles in preserving the intestinal barrier.[43] Together with the small intestinal niche, the mucosal microbial community is almost never sampled in diet–microbiome studies. Emerging evidence suggests that it is influenced by host diet, including fibre availability and overall diet quality,[44,45] and this may influence mucosal barrier function. This region may therefore be more important for the health implications of diet–microbiome interactions than previously recognised.

Understanding the microbial community structure

Methods of community assessment

The majority of diet–microbiome studies utilise amplicon sequencing of a segment of the 16S ribosomal ribonucleic acid (rRNA) gene, providing a rapid snapshot of the overall microbial community (Figure 1). Extensive, curated databases of 16S rRNA gene sequences are available that map sequences to the taxonomic assignment of organisms.[46–48] Amplicon-based profiling studies have typically grouped sequences into clusters (operational taxonomic units, OTUs) based on a set sequence similarity. However, this reduces resolution and makes comparisons across studies difficult because each analysis will result in a unique set of OTUs.[49] More recent bioinformatics developments have led to exact determination of amplicon sequence variants (ASVs). These techniques do not rely on clustering of similar sequences, but retain all unique sequences, removing only those determined to represent sequencing error.[49–51] This leads to improved resolution and reproducibility, with the same ASVs potentially identified across multiple different studies.

Despite these advances, the 16S rRNA gene approach has several limitations. Amplicon sequencing is based on only a short gene segment, which may not differentiate closely related organisms, and certain taxonomic groups may be under-represented depending on the hypervariable region of the gene used.[52–54] In addition, taxonomic assignment of partial-length 16S rRNA gene sequences is often inaccurate below genus level.[55] This approach also restricts analyses to identification of bacteria and archaea, although approaches to capture fungi[56] and viruses[57] are increasing in scope. Metagenomic sequencing, and the resultant generation of more complete genomic data, is able to resolve some of the aforementioned issues, as well as provide information on the potential metabolic properties of an organism based on presence of relevant genes[58–60] (Figure 1). However, metagenomic sequencing is more costly and analytically intensive. The use of this technique on a select number of samples in combination with 16S rRNA gene amplicon sequencing represents an alternative approach.

Reporting on taxonomy

Identifying the specific microbes that respond to dietary substrates is one of the cornerstones for understanding diet–microbe interactions and their implications for health. Sequence-based analysis of the microbiome, using ASVs/OTUs or metagenomics-based species identification, allows for very granular reporting, which is valuable given the metabolic variability of the microbial community. However, this level of detail also highlights the inter-individual variability of the microbiome from one human to the next. Reporting at higher taxonomic levels may overcome some of this variability, allowing key trends to be identified, although it will also mask potentially important detail because microbes within a taxonomic group can have different metabolic activities or differential responses to dietary substrates. Overall, there remains little consistency as to which classification or taxonomic levels are reported (e.g., ASV, genus, family and/or phylum); thus, synthesising the findings across studies remains difficult.[61,62] Generation of publicly available raw datasets provides scope for meta-analysis of data, regardless of original analysis techniques, metrics used or taxonomic level reported. These syntheses will be invaluable for establishing further consensus in terms of specific diet–microbiome interactions.

Shortcomings of relative abundance

A key outcome in microbiome studies is microbial relative abundance, which describes the ratios of microbes that make up a given community. However, without quantification of cell numbers, it is not clear whether change in relative abundance over time is driven by a change in absolute abundance of that specific microbe, or rather collinear relationships with other members of the microbial community.[63,64] Change in absolute abundance, as well as overall density of microbes in the GI tract, is more likely to reflect biologically relevant differences in functional outputs, such as how much of a metabolite is produced, although this is also influenced by gene/protein expression. Recently, techniques have been developed that provide quantitative data in combination with sequencing; for example, through the addition of known quantities of exogenous DNA[65,66] or the use of flow cytometry.[63,64]

The limitations of relative abundance are particularly important when considering responses to dietary substrates. These can take the form of 'consumption' type responses, where microbes can increase their population size when their preferred dietary derived substrate(s) are in excess. By contrast, those microbes with a 'limitation'

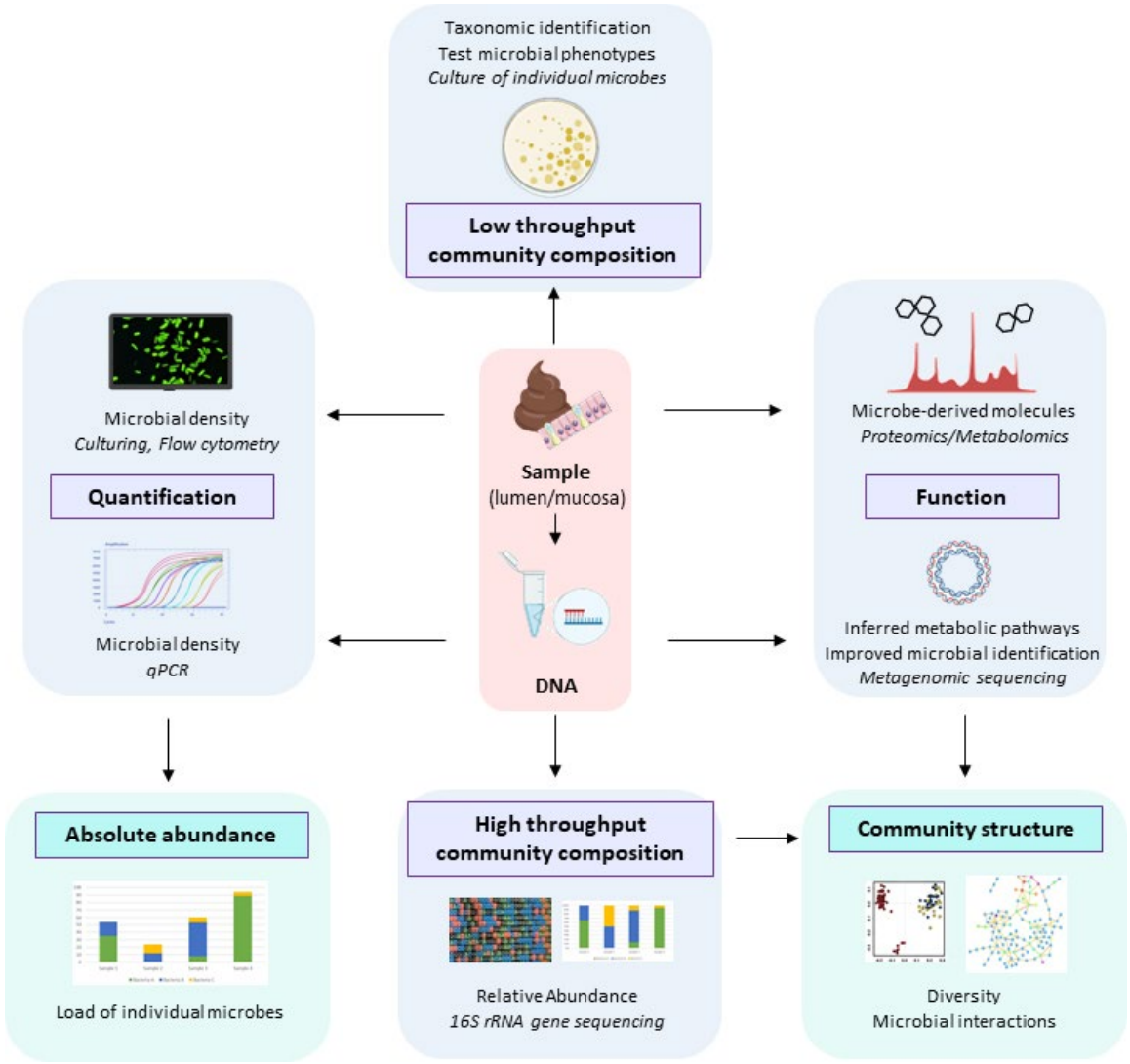

FIGURE 1 Overview of microbial analysis techniques and applications. The use of 16S ribosomal ribonucleic acid (rRNA) gene amplicon sequencing provides an overview of the microbial community and the analysis is based on relative abundance. Metagenomic sequencing, in which total genomic DNA is analysed, enables insight into potential functions encoded by microbes (e.g., substrate degradation and metabolite production). These microbial functions can be measured more directly from biological samples through techniques such as metabolomics and proteomics. Quantification of absolute abundance of microbes can be achieved directly by assessing cell numbers (culturing or flow cytometry), or through surrogate analysis of DNA, such as by a quantitative polymerase chain reaction (qPCR). Although the culture of individual microbes is low throughput, a renaissance in culture-based assessment of gastrointestinal microbes is being driven by sequence-based identification of new organisms, and is essential for fully characterising these organisms and their metabolic and functional properties

type response, are able to maintain their population when dietary substrates are limited, often as a result of the use of host-derived substances such as mucin.[67] Microbes may also appear to increase in relative abundance in certain circumstances, such as when fibre availability is low, but may actually have a stable population that becomes a proportionally larger component of the overall community because of the reduced abundances of other microbes.[68] Such collinear relationships are also complicated by microbial cooperation and cross-feeding, which is increasingly recognised as key to microbial community structure in the gut and production of health-associated metabolites such as short chain fatty acids (SCFAs).[69–71] An enhanced understanding of these relationships will be required for optimising intervention strategies that target the microbiome.[69]

Perspectives on diversity

Diversity within the microbial community of an individual (alpha-diversity) is a frequent outcome measure in diet–microbiome studies. Individuals following a traditional agrarian-style diet higher in microbiota-accessible carbohydrates harbour higher microbial diversity than those consuming a Western-style diet.[72–74] Despite this, short-term fibre supplementation alone does not increase diversity,[61] suggesting that habitual diet may be a more important determinant. Lower diversity is frequently associated with disease states, such as Crohn's disease and obesity, and, although

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meta-analyses may not always support this concept,[75,76] diversity is an endpoint in many diet intervention studies. Although greater microbial diversity is often considered an indicator of GI health, greater diversity may not equate to increased abundances of microbes that perform beneficial functions,[77] nor is it an absolute requirement for stability and resilience of the microbial ecosystem.[78] Therefore, although alpha-diversity is an indicator of microbiota structure, it is most useful when assessed in combination with other measures of the microbial community.

Assessment of beta-diversity, in combination with analyses such as principal coordinates analysis, allows for determination of global differences between samples, and can be based on the presence or absence, or abundance, of particular organisms (e.g., Jaccard's dissimilarity, Bray–Curtis metrics), as well as phylogeny (evolutionary relationships between microbes; e.g., UniFrac).[79,80] This has also led to the identification of characteristic microbial communities within the human population, termed enterotypes, which have been broadly linked to long-term dietary intake.[81] The presence of such differing community structures may be useful for stratification of patient groups and identification of individuals likely to respond to dietary interventions. However, the microbial networks and interactions, such as cross-feeding and competition that drive assembly of these communities, as well as the implications for nutrient utilisation, metabolite production and human health, remain to be completely understood. In this context, a focus on guilds or networks of co-abundant organisms that respond to the same dietary substrates, may be informative.[82]

Importance of microbial function

A consideration of taxonomy alone does not provide mechanistic insight into the effect of diet on the microbial utilisation of nutrients. This is because the metabolic functional properties of microbes are not necessarily linked to taxonomic identity, as per the ecological principles of functional redundancy and strain variation. Functional redundancy refers to taxonomically distinct organisms that possess similar metabolic properties. For example, distantly related *Bifidobacterium adolescentis* (Actinobacteria phylum) and *Ruminococcus bromii* (Firmicutes phylum) both have the ability to degrade and utilise resistant starch.[83,84] Therefore, it is unsurprising that an increased faecal SCFA concentration in response to resistant starch supplementation is associated with a greater abundance (relative or absolute) of different organisms across individuals.[83,85] By contrast, strain variation refers to closely related microbes with vastly different functional capabilities, as a result of varying gene content.[86] This is highly relevant in the context of the GI microbiota, as exemplified by *Escherichia coli*, for which there are both probiotic and pathogenic strains.

The direct measurement of metabolites in combination with characterising microbial profile may be more informative for health outcomes, enhancing understanding of how microbes respond to habitual diet and dietary interventions. A focus on specific microbial metabolites such as by-products of fibre fermentation (e.g., SCFA), choline metabolism (e.g., trimethylamine/trimethylamine *N*-oxide) and sulphur-containing compounds (e.g., hydrogen sulphide) has been extremely valuable for discovering associations between diet and clinical endpoints, such as intestinal barrier function or metabolic and cardiovascular health, and, in combination with microbial profiling, can explain inter-individual responses to dietary intake.[87,88] Metagenomic analysis assesses microbial gene content, and therefore provides not only information on taxonomic composition but, also some inference of microbial metabolic activity. Although metatranscriptomic analysis provides a direct assessment of microbial transcriptional activity, the very short half-life of microbial RNAs means that this is highly confounded by timing of sample collection.[89] Metagenomic and metabolite analyses are also constrained by the volume of microbial 'dark matter' that still exists. Many organisms remain uncultured and lack sequenced genomes, and many (possibly up to 70%) genes, proteins and metabolites have unknown functions.[90,91] The generation of readily accessible, integrated data that captures the metabolic properties and activity of the microbiome, especially where relevant to nutrient utilisation, will represent a key step forward in the field.

Analysis challenges

Collinearity

The issue of collinearity pervades both diet and microbial datasets. For diet, this is the notion that changing one component of the diet precipitates compensatory changes in others. This is less relevant in nutrient supplementation studies but is important in food or whole diet interventions. For example, a whole diet intervention aiming to modulate the microbiome through increasing intake of fruit, vegetables and wholegrain foods will likely result in a compensatory reduction in protein and/or fat intake, assuming that energy intake remains constant. This change in protein and/or fat intake will in itself have distinct effects on the microbiome. As an added complexity, these dietary changes will lead to higher fibre, polyphenol[92] and unsaturated fatty acid intake,[93] each of which specifically impact the microbiome. Hence, collinearity limits the degree to which specific microbial changes can be attributed to altered intake of individual nutrients in most dietary intervention trials. Despite this, comprehensive assessment of all relevant nutrients will assist in the interpretation of findings. Three-dimensional modelling, or 'nutritional geometry' to simultaneously assess combinations of dietary components may also prove useful in deciphering some of the complexity of diet–microbiome interactions in human studies.[94]

Sequence-based microbiome profiling studies, and other 'omics datasets, also result in large collinear datasets. This presents a variety of statistical challenges, and highlights the importance of involvement of an experienced biostatistician. Many commonly used statistical methods are not designed for proportional data.[95] There has been considerable

TABLE 2 Best-practice guidelines for diet–microbiome research

Study design • Hypothesis-driven (design all aspects of study to answer a question regarding a specific diet–microbiome interaction) • Power calculations; use largest sample size possible
Participant selection and characterisation • Thoroughly characterised study participants (e.g., anthropometric data, disease status and severity, medications, lifestyle factors) • Consider whether any factors warrant exclusion (e.g., recent antibiotic use)
Dietary data collection • Utilise expertise of a nutrition professional • Consider strengths and limitations of dietary assessment methods relevant to the research questions (see Table 1) • Align dietary data collection with microbiota sampling (e.g., one day prior to stool collection) • Employ strategies to reduce under-reporting and other recall errors
Biological sample collection • Select region of GI tract appropriate to research question (e.g., stool versus mucosa, small versus large intestine) • Align collection of microbiota and other biological samples (e.g., plasma) • Transport and store samples appropriately (preservative or cold temperature) • Ensure procedures are standardised for all participants to avoid methodological-induced variation • Collect multiple samples to address intra-individual temporal variability
Dietary analysis • Utilise expertise of a nutrition professional • Single nutrient versus global (e.g., diet quality) approach • Quantification of microbiota-accessible components (e.g., types of dietary fibre) • Collinearity (consider nutritional geometry)
Microbial community analysis • Develop analysis strategy with input of microbiome expert prior to commencing study • Consistency across all aspects of sample processing and analysis (e.g., DNA extraction protocols; inclusion of controls to account for reagent contamination and batch effects; consistent use of bioinformatics pipeline) • Select analysis method based on research question (e.g., metagenomic sequencing to identify capacity to utilise particular nutrients; direct measurement of metabolites) • Consider multiple methods of microbial analysis (e.g., microbial profiling with metabolite quantification) • Measure and report both relative and absolute abundance (e.g., using qPCR) • Consider the impacts of intra- and inter-individual variability when designing analysis strategy
Statistical analysis • Utilise expertise of an experienced bioinformatician for integration and analysis of diet–microbiome data • Data proportional / not normally distributed: transform data (e.g., centred-log ratio) • Sparse data (multiple zero datapoints): use statistical methods validated for microbiota data • Where there are many more outcome measures than number of samples: apply stringent correction for multiple comparisons • Multi-omics data: integrate datasets prior to analysis; use of modelling • Confounding effects: multivariate/mixed models • Utilise metrics and statistical tests developed specifically for microbiome analysis

Abbreviation: DNA, deoxyribonucleic acid; GI, gastrointestinal; qPCR, quantitative polymerase chain reaction.

debate over the normalisation strategies that should be applied to overcome this.[95–97] Importantly, data normalisation/transformation should be applied, such as centred-log ratio, when utilising parametric statistical tests. In addition, specific metrics developed for analysis of sequence-based microbial profiling data are available to assess changes in abundance,[98,99] correlations,[100,101] or to identify biomarkers,[102] and can complement standard statistical tests. When combining multiple large datasets (multi-omics), including dietary data, the statistical challenges are even more complex; however, method development is progressing in this area.[103]

Type-1 error and power calculations

Another major analysis challenge in cross-sectional studies is the presence of type-1 errors that result when a large number of outcome measures (e.g., hundreds of bacteria, multiple dietary variables) are derived from a small number of study participants. Correction for multiple comparisons, using false discovery rate or Bonferroni correction, can reduce the risk of type-1 error, although even these may be suboptimal as a result of the non-parametric and non-Gaussian distribution of microbiome data.[104] Hence, care must be taken not to over-interpret the significance of changes in abundance of individual OTUs/ASVs. Further complicating data analysis is the presence of 'rare microbes' that are present in some individuals but absent in the majority (i.e. zero-inflated data), increasing the risk of type-1 error[97]; however, stringent data filtering methods (e.g., only considering microbes present in at least 25% of participants) can help alleviate this problem.

Background dietary change during intervention trials is also a common problem when interpreting diet–microbiome findings. In well-powered studies, randomisation theoretically controls for this. However, under-powering is common in microbiome-targeted supplementation research, and

lack of dietary assessment creates uncertainty over whether microbiome findings are indeed a result of the effect of supplementation *per se* or, instead, are influenced by change in background diet secondary to the Hawthorne effect or other unintentional variability in dietary intake. More participants clearly provide enhanced study power, although the numbers required even for small effect sizes in diet–microbiome research readily reach thousands because of the inter-individual variability in the baseline gut microbiome.[76] Although there has been exploration of how to apply power calculations in microbiome profiling studies,[105,106] this is highly challenging when considering the microbiome as a complex ecosystem and the multivariate nature of datasets generated. Longitudinal studies provide a means to address some aspects of inter-individual variation, although many of the same statistical challenges remain. This highlights the utility of placing datasets from individual studies in context, such as through meta-analysis, to delineate trends from type-1 errors.[79,107]

Recommendations for best-practice study design

Despite the challenges of diet–microbiome research, best-practice study design (Table 2, Figure 2) can aid in overcoming many of the aforementioned challenges. Hypothesis-driven approaches are key, where studies are designed to answer specific questions regarding host-diet–microbe interactions or interventions are tested in specific contexts. Overall trial design and planning for congruence in diet and biological sample collection, along with a consideration of the analysis and statistical challenges highlighted above, will facilitate high-quality research and greater confidence in reported outcomes.

The study population

Clearly, characterising the study population is fundamental in diet–microbiome research. Confounding factors are particularly important to measure, some of which exert a stronger influence on microbial profiles than host genetics.[108] These factors broadly include ethnicity, anthropometric data, health status, primary disease and comorbidities, medication use, and lifestyle factors,[108–111] and may also serve as exclusion criteria, such as the recent use of antibiotics or probiotics. The microbiome composition in individuals with chronic disease, often characterised by reduced microbiome richness (such as in inflammatory bowel disease)[112] or temporal instability (such as in irritable bowel syndrome),[113] is often not mirrored in healthy individuals, who have relative stability of the microbiota over time.[114] In disease, and particularly in GI disorders, altered dietary habits may partially mediate this divergent response.[115] Hence, perhaps one of the most important characteristics to measure and report is participant disease status (e.g., relapse/remission) and severity.

Trial design

There are three major possible types of trial design in diet–microbiome research and, as in any other field, the choice of design should primarily be guided by the research hypothesis. First, cross-sectional studies measuring diet and the microbiome at one point in time allow for evaluation of the associations between these two variables. These studies are highly subject to external confounding and, although they have an implicit inability to establish whether the condition (microbiome profile) or the exposure (diet) came first, they are important in helping generate testable hypotheses.[2,3,116] Second, longitudinal studies that repeatedly measure diet and microbiome over time can provide deeper insight into diet–microbiome relationships because they allow measurement of the effect of temporal dietary variation on microbial composition and stability within an individual.[9] Third, intervention studies are those that supplement or restrict a nutrient, food or food component, or implement a 'whole diet' alteration (e.g., Mediterranean or Atkins diet) and are therefore able to demonstrate more direct influences of dietary variables on the microbiome. Confounding effects of habitual diet and baseline microbial communities remain a challenge in intervention studies; however, effects will become more completely understood as further RCTs recruit participants based on pre-defined background diet,[117] and we benefit from learnings of relevant longitudinal data.

Data collection: Diet

Timing dietary assessment so that it is immediately adjacent to biological sample collection is extremely important given the rapid effects of diet and other host factors on microbiome composition.[1,4,9] Of the multiple methods used to assess diet (Table 1), the 24-h recall or unweighed food record are typically used to measure short-term intake. Reducing the number of recording days may ease participant burden, although this must be balanced against the importance of capturing daily dietary variation, which is of particular importance in diet–microbiome research.[118,119] Importantly, short-term dietary data can also be used to confirm dietary composition remains stable throughout microbiome-targeted (e.g., probiotic or prebiotic) supplementation studies. Remarkably, assessment of diet is frequently overlooked in such trials, as highlighted in recent systematic reviews of fibre[61] and probiotic supplementation.[120]

Assessment of long-term dietary intake is also relevant, particularly in cross-sectional studies, and provides a broader nutritional context for how diet shapes overall community structure. Food frequency questionnaires (FFQ) are typically used for this, and nutrient-specific FFQs are also available, including some with high relevance to the microbiome, such as those measuring intake of prebiotic carbohydrates.[121] Validated culturally-specific questionnaires are also available, enabling capture of aspects of diet unique to

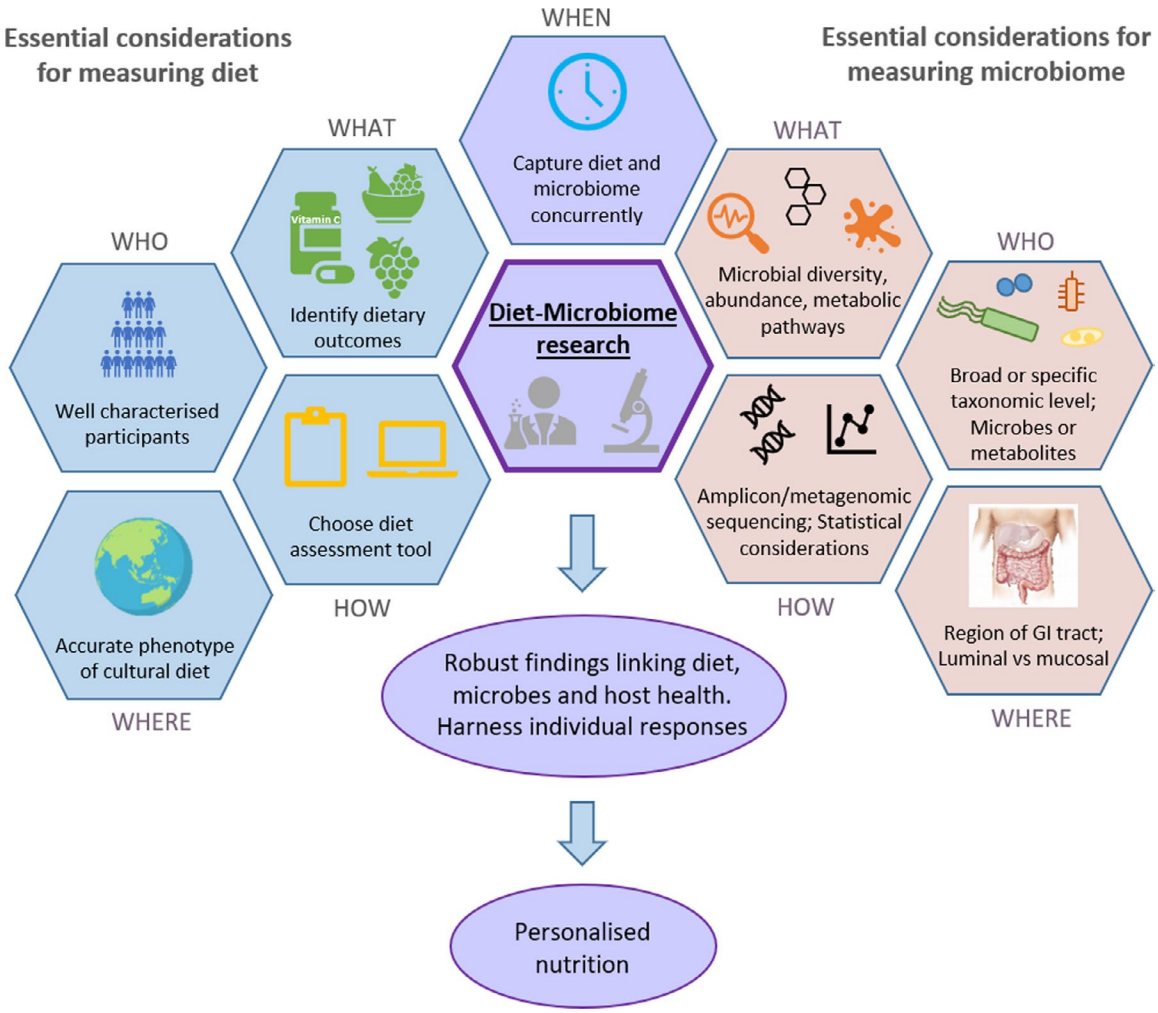

FIGURE 2 Summary of key considerations in diet–microbiome research. A robust study design includes a well characterised participant group, consideration of the availability of dietary substrates to microbes within intestinal niches, alignment of diet and microbiome data collection, rigorous choices of dietary and microbial assessment methods informed by experts, and appropriate statistical integration of these collinear datasets. GI, gastrointestinal

specific geographic regions. Broader measures of dietary composition, such as diet quality (Table 1), may also be applied as measures of long-term dietary intake[122,123] and, in contrast to measuring nutrients alone, provide a global assessment of diet as previously described. In addition to cross-sectional research, habitual long-term intake may also be of relevance in intervention studies as mentioned previously, helping to explain inter-individual variation in microbial response to certain dietary interventions.[117]

Data collection: Microbiome

Collection of samples for microbiome analyses and related downstream processes including storage, processing and analysis methods, should be consistently applied across all samples to avoid technical variation.[124] Although time for biological sample transport and storage prior to analysis is required, care should be taken to limit sample degradation by using timely and appropriate preservation methods.[125] If a large number of samples need to be processed in batches, or after different lengths of storage, controls (such as mock microbial communities) should be included to account for batch effects and accounted for during statistical analysis.[126]

As highlighted previously, the selection of suitable experimental analysis techniques for microbial samples, as well as the statistical approaches to be used and the metrics or outcomes to be reported, is crucial, and can potentially elevate (or appropriately downgrade) the significance of research outcomes. Ideally, a variety of microbiome analysis methods should be employed in concert with best-practice data analysis (Table 2), and these choices must be considered at the trial design phase prior to participant recruitment and sample collection. Example strategies could include complementary use of 16S rRNA gene amplicon sequencing for microbial profiling along with metabolite detection in the stool and blood[87]; or the addition of metagenomic sequencing to facilitate assessment of how functional redundancy shapes individual responses.[60]

CONCLUSIONS

A strong body of evidence exists regarding the importance of diet–microbiome interactions in facilitating health, or driving disease pathophysiology. However, the field continues to be dominated by a wealth of associative studies, fewer intervention trials and a lack of research providing mechanistic insights that identify the distinct diet-driven microbial alterations beneficial for human health. From a dietary perspective, tools that facilitate less onerous yet accurate estimations of dietary intake will be key. There is a need for the further development of methods that enable the measurement of nutrients in foods that are relevant to microbes (such as types of fibre and polyphenols), methods that estimate the availability of nutrients at the luminal substrate–microbe interface, and cost-effective methods that identify valid biological biomarkers of nutrient intake. From a microbiome perspective, improvements in our ability to measure and understand microbial metabolic properties, the response to nutrient availability, and interactions between microbes that drive community structure and function will move the field beyond taxonomic lists. Studies that investigate human diet–microbiome interactions using best-practice techniques, with collaboration across the disciplines of nutrition science and microbiology, are essential (Figure 2); this will drive research and clinical practice towards the goal of personalised nutrition recommendations for disease prevention, as well as the development of therapeutic diets for a broad spectrum of microbiome-associated disorders.

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CONFLICT OF INTERESTS

The authors have no conflicts of interest.

AUTHOR CONTRIBUTIONS

HS conceived the manuscript. All authors contributed equally to manuscript preparation and editing before submission.

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