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PERSPECTIVES

Considerations for best practices in studies of fiber or other dietary components and the intestinal microbiome

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dietary fiber; gastrointestinal; in vitro fermentation; microbiota; nutrition

INTRODUCTION

> "Life would not long remain possible in the absence of microbes."
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> –Louis Pasteur

Enormous progress in defining the human intestinal microbiome has been made over the last decade, in large part, beginning with the launch of the National Institutes of Health (NIH) Human Microbiome Project that has stimulated research support from other funders. Many papers and workshops have addressed a variety of best practices needed to improve analyses that are part of understanding the complex ecosystem in the gut, such as sampling, nucleic acid extraction, methods to identify microbes and their metabolites, and data reduction and analysis; these were specifically excluded from discussion at this workshop. However, there have been few attempts to standardize the dietary aspects of studies on the intestinal

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microbiome, whether in vitro, in animal models, or in humans. Most published studies report only minimal information on dietary components, even though there is substantial evidence that diet is a primary modulator of microbial composition. A workshop organized by program staff of the NIH and the U.S. Department of Agriculture, Agricultural Research Service, brought together 16 speakers focused on dietary aspects of microbiome studies. The purpose of the workshop was to improve rigor and reproducibility in research on the colonic microbiome and to identify important dietary information that should be reported and parameters to consider in design of research studies, particularly for clinical studies on diet and the intestinal microbiota. The workshop was held on the NIH campus on June 13 and 14, 2017, and was attended by ~100 people on site and >100 via webinar. Extensive time was built into the workshop schedule to allow discussion of topics on which there were no obvious solutions.

WORKSHOP SUMMARY

Focus on dietary fiber. The workshop's opening session focused on the structure of dietary fiber and other indigestible carbohydrates and their relevance to feeding the microbes in the colon. Estimates of macronutrients that are unabsorbed in the small bowel include 2–10 g/day of sugars, 3–9 g/day of protein, 8–40 g/day of resistant starch, 2–10 g/day of oligosaccharides, and 10–20 g/day of dietary fiber; the latter three food components are the dominant nutrient sources for the colonic microbiota, and all analyze as dietary fiber but are usually reported as distinct entities. Other potential nutrient sources for gut microbes include endogenous secretions from the gastrointestinal (GI) tract, such as proteins, enzymes, and bile acids (estimated at 4–6 g/day), mucin (2–3 g/day), and sloughed epithelial cells. The term "dietary fiber" has had multiple definitions over the last 60 yr, which has contributed to a lack of consistency in reporting. Even today, the Codex Alimentarius definition of dietary fiber includes carbohydrate polymers with 10 or more monomeric units that occur naturally in foods or are synthesized but are not hydrolyzed by the endogenous enzymes in the small intestine of humans and is accepted by 187 member nations. However, this leaves to national authorities the option of including fibers with degrees of polymerization between three and nine; carbohydrates with degrees of polymerization ≥10 are universally acknowledged as meeting the definition, but the assays to analyze them vary. For purposes of this discussion, the broader definition of dietary fiber is used. Although that includes oligosaccharides and resistant starch forms, those are identified separately, because they are almost always described as such in research studies.

The three-dimensional structures of dietary fibers are not uniform and have not been completely described. This is important for microbial access to these polymers and their susceptibility to fermentation. Despite many claims in the literature that several forms of fiber are not fermented, only lignin, isolated microcrystalline cellulose, and certain resistant maltodextrins are truly unfermentable by gut microbes. Within a plant, different types of tissues, such as vascular, parenchyma, mesophyll, and epidermis, contain different structural forms and types of dietary fiber that are connected to variable amounts of proteins with different constituent amino acids. These tissues are composed of different cell types, cell-wall thicknesses, amounts of lignin, and propensity toward being fermentable. The types of bonds and covalent or noncovalent linkages between fiber types and proteins are also remarkably variable. Whole grain foods contain resistant starch as well as fiber, but the physical process of milling grains has major effects on chemical composition and subsequent use.

There are at least four types of resistant starch differing in chemical structure, but all escape digestion in the upper GI tract and serve as nutrient sources for bacteria in the colon. Physical factors of dietary fiber that are relevant to bacterial fermentation include viscosity, gel-forming capacity, and water-holding ability. The terms "soluble" and "insoluble" fiber are useful from an analytical perspective; Koropatkin et al. (20) showed that soluble fiber is usually fermented in the proximal colon, and insoluble fiber is fermented more distally but usually not completely. Classifications based on the physicochemical properties of the fibers, including solubility, viscosity, and fermentability, which are related to the clinical outcomes of fiber consumption, such as laxation and cholesterol-lowering effects, are also relevant when considering diet-microbiota interactions, especially in human subjects. The complexity of secondary and tertiary structures of dietary fiber types cannot be overemphasized; secondary structure refers to the linear chains of carbohydrate moieties, whereas tertiary structure refers to the three-dimensional arrangement that may differ in which bacterial enzymes digest it. Fiber is the most chemically complex of any food constituent important in nutrition, which likely explains issues inherent to its definition and analysis. However, the knowledge of the amount and chemical type of fiber is insufficient due to the complexity of the diet matrix in which it is presented if we are to understand how the intestinal microbiome ferments various forms of dietary fiber.

There are hundreds, or perhaps thousands, of discrete fiber structures that align with bacterial digestive abilities. Discrete structures may provide competitive niches for different bacterial strains. For example, only some *Bacteroides* ferment complex arabinoxylans, whereas most do not. *Prevotella* have not been proven to ferment these complex arabinoxylans (9a). In addition to the naturally occurring nonstarch polysaccharides, nondigestible oligosaccharides, and resistant starch, novel carbohydrate compounds have been synthesized, such as polydextrose and mixed-linked α-glucans, which share lack of digestibility by mammalian enzymes but are partially degraded by gut bacteria. The potential exists to match fiber types/structures with bacterial strains to favor growth in the colonic environment. It may be possible to promote or suppress specific bacteria using custom-blended fiber formulations. Prebiotics are types of dietary fiber or resistant starch that stimulate the growth of beneficial bacterial species, such as lactobacilli or bifidobacteria.

Investigation of the genes for carbohydrate processing in *Bacteroides thetaiotaomicron* and *Bacteroides ovatus* reveals almost 2,000 genes that code for ~650 enzymes, far more than exist in mammalian systems (24), allowing those two species to degrade nearly all common glycans derived from diet or host secretions. Use of a synthetic gut microbiota in gnotobiotic mice, comprised of 14 commensal bacterial species, demonstrated that when fermentable fiber is not included in the diet, colonic mucin is preferentially used (12). Within 2 weeks,

significant declines in *B. ovatus* and *Eubacterium rectale* are noted, with large expansions of *Akkermansia muciniphila* and *Bacteroides caccae*, which preferentially degrade mucin, associated with thinning of the protective layer in some but not all studies, exposing the colonic epithelial layer to the opportunistic pathogen, *Citrobacter rodentium*, and subsequent morbidity and mortality. Some of the relevant questions related to these observations were the following: *1*) whether gnotobiotic animals with defined microbiota are valid models of animals with natural microbiota; *2*) whether humanized mice were more realistic; *3*) whether those with conventional microbiota exhibit colony-to-colony variation; *4*) whether an optimal diet for animals in such studies is a natural diet or a fiber-free base to which defined fibers are added and how such diets should be processed and cooked to mimic the human food supply; and *5*) the causes of variation in both animal and human microbiota, including both diet and host factors. Most of the answers depend, in part, on the experimental question being asked, but currently, there are no clear answers to any of these issues. Thus a best practice is for investigators to provide as much detail as possible about feeding and housing conditions of test subjects, even if only as online supplemental material for their papers.

Other dietary factors. As noted above, macronutrients are not completely assimilated in the small intestine, so some provide fermentable substrate in the colon. In addition to polysaccharides, non-nutrient food constituents can modulate the composition and activity of the colonic microbiome. Among the more prominent food-derived factors are the polyphenols. Numerous polyphenols are obtained from coffee, tea, wine, fruits, and vegetables; berries are especially rich sources and can contain various classes of polyphenols, such as tannins, flavonoids, anthocyanins, and proanthocyanins. For example, a polyphenol-rich extract of cranberry added to a high-fat, high-sugar diet fed to mice led to modified gut microbiota, reduced intestinal inflammation, insulin resistance, and obesity compared with mice that only received the high-fat, high-sucrose diet (1). Other modulators of the gut microbiota are proteins and omega-3 polyunsaturated fatty acids; these dietary components also improve glucose tolerance, inflammation, and dyslipidemia in mice (10). Most, if not all, rodent studies using purified diets have casein as the sole dietary protein source, which is practical but not at all relevant to the human diet that contains a mix of proteins. Any purified protein or mixture of them can be used in animal diets, but the ideal depends on the question being asked and is affected by availability, purity, and cost. In addition to digestible and fermentable food components, several food additives have been studied in mouse models to examine whether they alter the microbiota. Part of the rationale for these studies is the large geographic variation in prevalence of inflammatory bowel disease (IBD), which may result from changes in the microbiome due to variable exposure to environmental factors, including diet. Whereas many nondietary, hygiene-related factors vary geographically, some food additives are widely used and could modify viability of gut bacteria. For example, the emulsifiers Polysorbate 80 and carboxymethylcellulose, which are detergent-like molecules that stabilize mixtures of immiscible liquids, alter the mouse intestinal microbiota in a detrimental way, promoting colonic inflammation and metabolic syndrome, although the extent to which the experimental administration of these compounds in feed or drinking water mimics human exposure to these compounds is unclear (6). These compounds also increase lipopolysaccharide and flagellin concentrations in feces from mice while thinning the colonic mucin layer, leading to colitis in genetically susceptible animals and to low-grade intestinal inflammation in unimpaired hosts. These changes were associated with increased adiposity, with those differences eliminated in germ-free mice. Such detrimental effects were observed even at doses thought to mimic reasonably the overall consumption of emulsifiers by people who eat many processed foods. The two emulsifiers were studied for effects on the human microbiome in the mucosal simulator of the human intestinal microbial ecosystem, and the new steady-state microbiotas were transferred to germ-free mice. Recipients of the emulsifier-treated in vitro microbiotas gained more weight, were glucose intolerant, and had significantly shorter colons with thinner mucin layers, allowing bacteria close access to the epithelial lining (9). The latter observation also was seen in humans with type 2 diabetes; distance of bacteria from the colonic epithelial layer significantly correlated with measures of glucose intolerance (8). By highlighting the importance of diet/microbiota interactions in health and disease, semi-purified diets containing fat at the same concentration as in chow, but devoid of fermentable fiber, led to higher weight gain and reduced large bowel mass (7). Conversely, the supplementation of a high-fat diet with the fermentable fiber inulin but not the poorly fermented fiber cellulose resulted in a microbiota-dependent fortification of the mucosa that prevented microbiota encroachment and protected against high-fat, diet-induced metabolic syndrome. Hence, not only higher fat but also absence of fermentable fiber can render a diet obesogenic. However, whereas the enrichment of a high-fat diet with inulin prevented high-fat, diet-induced gut atrophy and greatly reduced metabolic syndrome, mice consuming inulin-enriched purified diets of low- or high-fat content developed extremely severe colitis upon exposure to the chemical dextran sulfate sodium (27). Thus whereas strategies to enrich foods with fermentable fiber may hold long-term potential to induce a more beneficial microbiota composition, at present, our relatively poor understanding of how various dietary components impact both the microbiota and host in a range of contexts currently precludes certainty for the goal of promoting gut health.

Focus on rodent models. A vast array of experimental diets is fed to a large range of animal models. Most common diets are closed formulations (the percent of each ingredient varies to provide minimal variation in nutrient content) that vary in composition from batch to batch while meeting macronutrient requirements and are usually described as "chow" diets or natural ingredient diets. Another choice is open formula diets, such NIH-31, which do not vary in ingredient composition from batch to batch but may have slight variations in nutrient content. Open formula diets can range from those that are composed primarily of grains and other so-called "natural" ingredients to semi-purified diets, such as the American Institute of Nutrition (AIN)-93 formulations that are made from clearly defined ingredients, such as casein, corn starch, and soybean oil. Although AIN-93 is considered the gold standard in nutritional studies with rodent models, it was developed to prevent nutrient deficiencies, but the main criterion was maximum growth. That diet contains no fermentable dietary fiber

or other macronutrient that is intended to resist digestion in the small intestine. For this reason, the concept of microbiota-accessible carbohydrate (MAC) is important to define those foods or nutrients that fuel the gut microbiome (38).

Because of reduced diversity in the gut microbiota among Western populations compared with those eating more traditional diets, an animal model was developed to understand this potentially important observation. Humanized mice were fed a high- or low-MAC diet. Those on the low-MAC diet were maintained for four generations. Changes in the gut microbiota were reversible during the first generation, but feeding the low-MAC diet resulted in increasing loss of diversity that was not reversed by switching the animals back to the high-MAC diet at the fourth generation, although a fecal transplant, along with the high-MAC diet, was able to restore diversity (39).

The question of whether standard rodent chow is an appropriate control diet in gut microbiome studies was considered. Chow or standard chow refers to a variety of commercially available closed or open formula animal diets obtained by an animal facility in bulk to feed its animals. These diets are usually economically priced, low in fat (5–10% of energy), and optimized for protein and other nutrients for healthy growth of each species (rodent chow, monkey chow, etc.). It is tempting to use chow, because it is inexpensive and generally results in excellent metabolic health of most laboratory animals, including moderate weight gain and low concentrations of plasma lipids, glucose, and insulin. Chow feeding also results in the highest cecal and fecal weights, along with the highest concentrations of short-chain fatty acids (SCFAs). Data were presented that recovery of some bacterial orders was uniquely influenced by chow diets, especially Anaeroplasmatales and Verrucomicrobiales. Since there are likely differences in the gut microbiota with weight status in humans and rodents, clear answers are not yet available on whether weight gain is an independent variable for changes in bacterial content or function. Likewise, the effects on the microbiota of diet sterilization (animal diets are not sterile) by autoclaving or irradiation that affects the tertiary structure of starch and protein are not known. Furthermore, whereas we know that there are different growth patterns of some inbred strains of mice and rats, we do not have data on whether strains differ in their microbiota composition or metabolic patterns. Finally, we do not have adequate data on organisms other than bacteria, such as archaea, fungi, viruses, parasites, and bacteriophage or many of their functional capacities.

Nonrodent models. The use of nonrodent models for GI microbiome research was considered. Because of the large cecum in all rodent species and their coprophagy, there are likely fundamental differences in microbial activity and fiber requirements that differ from humans. Larger animals, such as pigs and dogs, also have the same key advantages of more commonly used rodents—short generation time, access to intestinal contents and tissues, relevant disease models, and control of experimental variables—but have GI physiology and anatomy, as well as omnivorous eating patterns closer to that of humans. In addition, these larger animals are, like humans, susceptible to diarrhea, which is rare in rodents. Primary advantages of the swine model include similar developmental stages to human infants, large litters, short generation times, and germ-free possibilities. The size of pigs allows for easier surgery if needed and many sampling options, including cannulation, at different sites on the GI tract. Additionally, some strains of pigs, such as the Ossabaw mini-pigs, naturally become obese and display the diagnostic criteria for metabolic syndrome. Limitations to the use of swine are their large size and expense and the relative paucity of laboratory facilities for housing them.

The dog is one of the most commonly used nonrodent models in biomedical research, particularly in pharmacological studies. Dogs become obese and display associated pathologies, as well as IBD, cancers, and other chronic conditions. Their genome is well characterized, and the diversity among breeds can be a strength. Canine IBD shares many similarities to the human disease with decreased alpha-diversity of the microbiome, decreased Faecalibacteria, and increased Gammaproteobacteria and *Escherichia coli* (41). The dog's microbiome displays similarity of microbial taxa, functional capacity, and activity to that of humans (40) with similar responses to inclusion of dietary fibers or prebiotics. Potential drawbacks to use of the canine model are their size, expense, expertise needed in handling, and animal welfare concerns.

Focus on in vitro systems for the study of the gut microbial ecosystem. Several systems are available, including simple batch fermentation, single-stage bioreactors, multistage bioreactors in which each stage models a different compartment of the GI tract (e.g., simulator of the human intestinal microbial ecosystem), and miniaturized systems, such as minibioreactor arrays or HuMIX (a microfluidics-based, human microbial coculture model system), which allow volumes of 15 ml or 400 μl, respectively. Depending on the question asked, one may select inocula of bacterial monocultures, defined mixed populations (which are usually limited in number of species but may have >80), or whole fecal microbiota. Several strengths of in vitro models exist, including minimal ethical constraints, experimental reproducibility, sampling accessibility, absence of host factors affecting bacterial viability (which also may be a limitation), and lack of absorption of metabolites, making them easier to measure. Key challenges of these models include the choice of the best inoculum; nature of the nutrient supply; emulation of the mucosal, as well as luminal, environments; lack of host components, including immunoglobulins; and need for high water content to allow flow through the system. A number of variables come into play in trying to emulate natural conditions, including temperature, which is known and easy to control; flow rate and pH, which both vary but generally require an average of known values; oxygen tension, which is very low in a healthy colonic lumen but may be fairly high near the mucosal surface and is usually controlled by the displacement of oxygen with nitrogen; water content; and food/nutrients, which are very complicated to mimic natural conditions.

The issue of inoculum is very important since feces are generally used to seed such systems, but such samples may be only representative of the distal colon. Other regions of the GI tract can be sampled by colonoscopy or endoscopy; patients with stomas, such as ileostomies, can be sampled, but there is always the concern over whether these are representative of the healthy population, and the issue of oxygen tension under these conditions is also a factor for consideration. Nevertheless, it appears that bioreactor contents may be more representative of the resident colonic bacterial community than that of feces (26). Because gut microbiota differ from individual to individ-

ual, it is difficult to ensure experimental relevance if a small number of donors are used. Some studies have suggested that fecal inocula can be pooled to create an average ecosystem, but this is controversial, with some data suggesting that one donor's microbiome outcompetes another's. Relevant to this point is the estimated diversity of the colonic microbiota. Whereas it is often stated that >1,000 species exist within each person, this number has been challenged, and there may be only 100–200 species per person (2). Just as the total number of colonic bacteria was cited in the past as being 10 times that of human cells, a more accurate estimate is likely equal in number (33). Important to studying this complex ecosystem is the low abundance of some strains, particularly those associated with the mucosa that may not be well represented in fecal samples. Additional data describing the full phenotype of the donor will also be helpful in establishing factors that matter for comparing data from different donors, such as dietary habits, health status, and drug or dietary supplement exposure.

The feeding of in vitro systems is virtually always designed by microbiologists and not nutritionists. To represent nutrient availability best in the colon, bioreactors need a medium that represents chyme from the terminal ileum. Most media do not accurately reproduce what is in the digesta. Furthermore, some microbes are adapted to niches in the colon, such as association with the mucin layer (25). Mucin can be added to the system to enhance growth of mucin-associated species, such as *A. muciniphila*. Some believe it is important to allow development of a steady state, often after a period of weeks, but others use models, such as the TNO intestinal model 2, that use dialysis to maintain physiological concentrations of metabolites, such as SCFA, eliminating the need for a steady state and thus designed for shorter, often 3-day, running cycles (43).

When asked "How many investigators in the audience of the workshop report that their fecal donors follow a typical diet and have not taken antibiotics for some period of time?" most attendees responded that was their practice. This demonstrated the general lack of reporting detail for this parameter, given the large number of dietary compounds that have the potential to affect growth of the GI microbiota. The provision of a dietary and medical history of fecal donors should be standard practice, perhaps as supplemental online material. There is substantial interlaboratory variability in results of fecal fermentation studies when well-characterized, single types of resistant starch are added. Much of this variation can be attributed to the diet and consequently, different microbiota of fecal donors. Butyrate, produced in vitro, correlates strongly with several nutrients available from plant foods, including dietary fiber, plant protein, and several micronutrients. Not surprisingly, those correlations can be observed with fermentation by specific bacteria, such as *Faecalibacterium prausnitzii*. However, it is rare to report diet of fecal donors other than that they followed an unspecified "average" diet, even though there is strong evidence pointing to this factor having major influence on the results (48). In addition, the occurrence of obesity in donors appears to be a substantial factor in the ability of fecal samples to produce butyrate following in vitro incubation (47).

Focus on human studies of the colonic microbiome. Numerous challenges in characterizing the effects of diet on the human gut microbiome were highlighted, including poor adherence to specific diets, relatively inaccurate characterization of dietary intake and composition, the reciprocal nature of dietary changes when isocaloric conditions are maintained, high costs, and other logistical challenges in completing either controlled feeding or large cohort studies. In addition, human observational studies generally point out associations so that a combination of reliance on model systems along with controlled feeding studies of volunteers is needed. Therefore, reasoned use of a combination of human and animal model studies may prove more informative than either approach alone.

In addition to the microbial diversity among humans noted above, there are seasonally stable and seasonally labile species identifiable in feces from the Hadza of Tanzania, the last true hunter-gatherers in East Africa (37). A potential result of the lower diversity in Western populations is reduced metabolic output from the gut microbiota; this has been shown for SCFAs, but there may be other biological mediators absorbed from the colon that interact with receptors on cells beyond the intestine. Better tools are needed to study how individual bacterial strains establish a geographic niche in the gut. One successful approach is the use of a novel phage promoter and fluorescent protein expression in various species of *Bacteroides* that allow quantitative identification and differentiation of strains within the gut, down to the level of colonic crypt occupancy (42). Although fermentable substrates, such as inulin, can drive increases in specific bacterial species, the ability of a single strain to colonize a pre-existing microbiota is variable. An example of the ability of ingested bacteria to transfer genes to microbiota in the human gut is for digestion of sulfated polysaccharides, specifically the porphyrans and agar found in seaweed (18). This phenomenon is exemplified by differences in the fermentability of these substrates by Japanese versus North Americans. Japanese eat ~14 g/day of seaweed, and their microbiota can ferment these polysaccharides, whereas North Americans, who generally do not consume seaweed, cannot ferment these substrates. These observations suggest that the ability to use polysaccharides from other food sources can be engineered into the human microbiome.

Whereas changes in gut bacteria have been reported among subjects who are at healthy weight, obese, or following bariatric surgery, the observed modifications are not always reproducible. Animal models and controlled feeding of humans can be used to address these phenomena to provide clear answers in adequately powered studies. Germ-free mice are resistant to obesogenic diets, and hepatic gene expression is markedly different in germ-free versus conventional mice (21). Among these differences are genes responsible for circadian rhythm signaling. The mouse microbiome exhibits diurnal oscillations that are influenced by high- and low-fat diet; this observation is consistent with diurnal fluctuations in cecal concentrations of metabolites, such as butyrate and hydrogen sulfate. These metabolites, in turn, alter hepatocyte circadian clock function and responsiveness within areas of the central nervous system. Timed administration of butyrate injected intraperitoneally can correct high-fat, diet-induced hepatic circadian dysfunction and obesity, suggesting that a modulating role of the gut microbiota may be sample time dependent. The conclusion from these observations is that the low-fat diet results in normal microbiome signals leading to both normal circadian functions and body weight, whereas the high-fat diet results in aberrant microbial oscillations and signals that lead to circadian disruption and

obesity (21). Germ-free animals lack microbial signals no matter which diet they are fed but still have circadian disruption and fail to become obese on a high-fat diet. All of these data suggest that the recording of the time of fecal collection in both animals or humans is needed.

A cross-sectional study of vegans and omnivores assessed diet with repeated 24-h recalls before sampling of plasma, urine, and stool for metabolomics and microbiota (46). Contrary to what most would have expected, higher consumption of fermentable substrates by vegans was not accompanied by increased concentrations of fecal SCFA, which was confirmed in a 10-day controlled feeding study. Of course, fecal concentrations of SCFA may not reflect in vivo exposure well since 95% or more are absorbed before elimination of stool. In a study of 98 subjects, fecal microbiota were clustered into enterotypes distinguished primarily by *Bacteroides* and *Prevotella* (45). These enterotypes were associated with long-term diet, particularly protein and animal fat (*Bacteroides*) versus carbohydrates (*Prevotella*). The vegans and omnivores had considerably different plasma and urinary metabolomes, but there were not huge differences in their microbiota, including measures of diversity. Intersubject differences in humans appear more important than many other factors, and this remains unexplained. A subset of subjects fed controlled diets that varied in fat and fiber maintained enterotypes but exhibited significant microbiome changes. Multiple studies implicate factors in both the diet and microbiome in IBD. Defined formula diets for treatment of Crohn's disease are most effective when they completely replace the normal diet of patients (22). Numerous metabolic changes result from alterations in the gut microbiota, not just from fermentation of indigestible carbohydrates but also from metabolism of minor amounts of other macronutrients, micronutrients such as choline, and conversion of bile acids from primary to secondary metabolites. In addition, many of the microbial metabolites are absorbed from the colon and circulate in the plasma, affecting target tissues in extraintestinal organs. This observation suggests the novel therapeutic modalities of either engineering the microbiota or feeding defined diets that alters production of small metabolites in the colon.

Presently, there is no biomarker available that reflects dietary fiber intake, so researchers rely on dietary histories that are often unreliable. The current Dietary Reference Intake recommendations for fiber are based on prevention of coronary heart disease from three observational cohort studies and are set at 14 g $\cdot$ 1,000 kcal^{-1} $\cdot$ day^{-1}, which translates to 38 g/day for young men and 25 g/day for young women, far more than current consumption in most Western countries. Studies aimed at increasing dietary fiber intake to 35 g/day have failed to prevent recurrence of adenomatous polyps (34), but experimental studies have shown that there is a threshold level for fermentation products derived from fiber, specifically butyrate that suppresses epithelial cell proliferation and tumorigenesis. The fiber/resistant starch concentration in the traditional African diet is >50 g/day and is associated with low colon cancer risk (29). Although the Polyp Prevention Study failed to show any difference in recurrence of adenomas with dietary intervention up to 8 yr, subgroup analysis showed a significant reduction in recurrence of advanced (>1 cm, >25% villous, or high-grade dysplasia) polyps in those consuming the highest quartile of high-fiber beans (30). With this background, a study was planned to compare a typical American diet with a traditional African diet in both native Africans and African-Americans for effects on fecal bile acids, SCFA, and biomarkers of colonic mucosal proliferation (29). The African diet provided 55 g/day fiber and 41 g/day fat, whereas the African-American diet contained 7 g/day fiber and 145 g/day fat. There is a >10-fold higher risk of colon cancer in African-Americans than in rural South Africans. The switching of diets resulted in marked reciprocal changes in SCFA, secondary bile acid formation, and markers of epithelial cell proliferation. In addition, the dietary switch resulted in noticeable shifts in the composition of the microbiota, with *Prevotella* dominant while following the African diet and *Bacteroides* dominating while following the African-American diet (30). Since diet can influence the relative concentrations of certain bacteria in the colon, it is relevant that a systematic review of both humans and animal models evaluating the microbiota and colorectal cancer concluded that the presence of the disease is associated with consistent increases of species members from *Fusobacterium*, *Alistipes*, *Porphyromonadaceae*, *Coriobacteridae*, *Staphylococcaceae*, *Akkermansia*, and Methanobacteriales, whereas others that were consistently decreased were *Bifidobacterium*, *Lactobacillus*, *Ruminococcus*, *Faecalibacterium*, *Roseburia*, and *Treponema* (3). In addition, bacterial metabolites of amino acids were increased, and butyrate was decreased in association with colorectal cancer in both humans and animals. Although more studies are needed, current data collectively suggest that at least 50 g/day of fermentable substrate is needed to reduce risk factors for colon cancer.

A number of issues are routinely ignored in studies of the human intestinal microbiota. The digestive tract is an open system, so dietary effects should never be discounted. Samples from the colon are essentially snapshots that do not distinguish between permanent and transient resident strains. Since it is generally assumed that each individual has his/her own microbiota, a large number of samples are likely needed to reach valid conclusions, and the effect of any dietary intervention will depend on the individual's baseline microbiota. Whereas diet explains ~60% of the variation in the gut microbiome of mice due to standardized feed and environmental conditions, it only explains ~10% of the variation in humans (31). Those differences may result from genetic differences, level of control over diet and environment, amount of fiber used, sample collection methods, and other variables. This observation raises the issue of whether researchers have relied too heavily on rodent models in predicting efficacy in humans. Although it is logical to focus on dietary fiber and other sources of fermentable carbohydrates, ~10% of ingested protein reaches the colon, and many bacterial metabolites of its fermentation, such as sulfides, phenols, polyamines, and ammonia, are harmful to the mucosal tissue. Diets high in protein or fat are necessarily low in both digestible and nondigestible carbohydrates, making it almost impossible to attribute causality to an individual macronutrient. An analysis of stool microbiota in monozygotic twins showed that intakes of energy, unsaturated fatty acid classes, and soluble fiber affected microbiota composition, particularly for *Bacteroides* spp. and *Bifidobacterium* spp., but body mass index was not a factor (36). The effect of diet is not the additive effect of individual nutrients, but diet needs to be considered as a totality. An unresolved question is whether a group of human subjects fed the same foods for perhaps 1 week

would show reductions in the marked interindividual variability alluded to above. A related question is whether dosage of dietary fiber should be provided in equal amounts daily, based on energy intake, metabolic body weight, or some other factors. There are currently no data to address these issues. Although such a dietary approach would be of limited generalizability to a diverse population, it would answer an important question.

Diet is a highly complex exposure, providing thousands of compounds in complex mixtures, and food structure, especially of plants, is rarely captured but may be important in determining what reaches the colon. Researchers often consider dietary intake in different ways, such as by individual foods or classes of foods, individual compounds, or dietary patterns. Controlled feeding studies use diets prepared to exact specifications and are the strongest in measuring a biologic effect of a dietary manipulation, but these studies are not designed to evaluate application of that dietary intervention under real-world conditions and are the most expensive to conduct. These studies can be conducted in parallel arms or with a crossover design; under the latter, all subjects receive all interventions, and each person serves as his/her own control. In crossover designs, it is essential that the washout period between treatments is long enough to be certain that the gut microbiota returned to its initial composition, but that time period has yet to be established, although it is likely at least a couple of weeks, based on typical GI transit times. These conditions allow tight dietary control, potential to test dose-response relationships, and monitoring of intermediate biomarkers, but limitations include a relatively short-term intervention, high expense, and inability to evaluate hard disease end points. One example is a study of feeding cruciferous vegetables containing glucosinolates and dietary fiber to alter the gut microbiome that used a low-fiber, low-phytochemical basal diet for comparison with the cruciferous-rich diet that provided high amounts of both compounds with each diet fed for 2-week periods (23). Each diet resulted in different fecal bacterial communities thought to influence metabolism of these bioactive food components. Although many individual nutrients may affect the intestinal microbiota, use of dietary patterns addresses some of the limitations of the single nutrient approach, including the high correlation among intakes, multidimensional aspects of foods, and inclusion of nutrients and non-nutrients. Short-term feeding of animal- or plant-based diets substantially alters the fecal microbiota but does not overcome large interindividual differences in microbiota composition (11). The animal-based diet increased several species as proportions of the total sequence count that are bile tolerant, since that diet also provided triple the fat of the plant-based diet, whereas fiber was approximately one-third that of the plant-based diet.

In addition to undigested carbohydrates reaching the colon, 5% of ingested fat, 8% of ingested protein, and unabsorbed vitamins, minerals, and phytochemicals all have potential to alter the bacteria in the large bowel. Most GI transit time is accounted for by transit within the colon, where water and electrolytes are absorbed, and bacteria grow on unabsorbed nutrients, as well as sloughed epithelial cells and mucin. However, we do not have much basic information on how transit time affects composition or function of the colonic microbiota, except that longer transit time is associated with increased microbial richness (32). Up to 80 g/day of resistant starch and 10–15 g or more of fructooligosaccharides have been tested to show a lack of GI side effects (17). Recent research on fermentable oligo-, di-, and monosaccharides and polyols has shown a reduction of symptoms in some people with irritable bowel syndrome following avoidance of those fermentable substrates (35). There is concern that this dietary approach greatly restricts dietary fiber intake and the healthier foods providing that nutrient. Although there is much interest in the health benefits of prebiotics, there are no prospective cohort studies linking changes in the fecal microbiota with health end points. The definition of prebiotics, proposed in the mid-1990s, was limited to increases in only a limited number of bacterial strains, but that was before nucleic acid-based analyses were available. It is now appreciated that other bacterial changes may be induced and that those must be tied to health benefits of the host (4), but an updated consensus statement was published recently, suggesting such modifications (16). Whereas increased butyrate is generally accepted as beneficial, and some intermediate markers are improved, it has not been definitively linked to a specific hard disease end point. Likewise, soy feeding has been studied for multiple benefits, but recently, it has been demonstrated to increase bifidobacteria and lactobacilli, as well as to alter the Bacteroidetes-Firmicutes ratio (19). Other dietary factors that alter the gut bacteria in humans include iron, ellagitannins, and flavanols; far more compounds have shown such activity in animal models. These include conjugated linoleic acid, proanthocyanidins, polyphenols, L-carnitine, flavanols, sphingomyelin, resveratrol, zinc, aspartame, saccharin, sucralose, carboxymethylcellulose, and polysorbate-80. Several nondietary factors affect transit time and laxation that in turn, might influence the microbial composition of feces; those factors include stress, exercise, smoking, coffee consumption, and personality. More accurate assessment of dietary exposure is needed, along with standardized methods, for the field to advance. All of these factors should be recorded by investigators, since they may reveal common influences on fecal microbiota.

Legal issues. Regarding regulatory considerations for studies of diet and the microbiome, the microbiome is a variable in studies of diet and health that may complicate interpretation of results. The regulatory framework relevant for microbiome studies includes consideration of intended use, safety, and labeling of the product. As for almost all food substances, the Federal Food, Drug, and Cosmetic Act, as amended, and related statutes, along with the Code of Federal Regulations and guidance issued by federal regulatory agencies, control the playing field. Probiotics—those species of bacteria considered to have some health benefit—are not defined as a regulatory product category and may be considered a food, dietary supplement, or a drug, depending on the intended use of the product. If the product is intended to treat, cure, mitigate, or prevent disease, the U.S. Food and Drug Administration (FDA) views it as a drug, and it is therefore subject to the premarket approval process. Several criteria differentiate how an ingredient is classified by the FDA (13). Mandatory food labeling informs the consumer about the basic nature of the food, its ingredients, the nutritional attributes, and other essential information, such as a warning about allergens. Although certain types of claims can be made voluntarily about a product, all labeling for which the manufacturer is responsible must be truthful and not misleading and conform to the

relevant statutes and regulations. Nutrition-related claims include structure-function claims (about maintaining health), nutrient content claims (about nutrient profile of a product), or health claims (disease risk-reduction claims for a food substance). Nutrient content claims and health claims require evaluation by the agency before use in food labeling. Unauthorized health claims may be considered unapproved, new drug claims if the substance purports to treat, cure, mitigate, or prevent a disease or health-related condition. For health claims, including qualified health claims, the FDA has provided guidance on how it reviews evidence (15). Studies suitable to the FDA are human intervention or observational studies; reviews, meta-analyses, animal, and in vitro studies are not. Human studies must have a control group, relevant statistical analysis, control of key confounders, validated biomarkers, and representation of the healthy population. The proposed, new definition of dietary fiber from the FDA includes nondigestible soluble and insoluble carbohydrates with three or more monomeric units and lignin that are intrinsic and intact in plants or isolated or synthetic, nondigestible carbohydrates with three or more monomeric units determined by the FDA to have physiological effects that are beneficial to human health. Consequently, isolated or synthetic, nondigestible carbohydrates that have not been determined to have a physiological effect that is beneficial to human health are not considered in the total fiber content of a product nor as the basis for a nutrient content claim. To date, the FDA has used lowering blood pressure, blood glucose, or cholesterol; improving laxation and bowel function; increasing mineral absorption; or reducing energy intake as physiological benefits to health to accept isolated or synthetic sources of dietary fiber for inclusion on the label. However, fermentation or effects on the microbiome have not been considered a physiological effect, because their beneficial effect on human health has not been established (14).

CONCLUSIONS

> "If you don't like bacteria, you're on the wrong planet."
> –Stewart Brand

The diversity of views expressed by speakers and audience discussants indicated that there are few clearly established best practices for design of studies of the intestinal microbiome in which diet is a main variable, but several best practices are suggested (Table 1). Lack of consensus on many potential best practices means it is incumbent on researchers to provide enough details about food and nutrient intake for animals or

Table 1. *Key points in design and reporting of diet in studies of the intestinal microbiota*

	Strengths	Weaknesses	Best Practices
General aspects of fermentable substrates	Should define fiber or resistant starch fully, including structure and particle size; provide trade name.	Listing cellulose, for example, is insufficient; there are dozens of varieties that differ in multiple characteristics.	Provide as much detail as possible; consider online supplemental description.
Animal studies	Mice most commonly used; can be conventional, gnotobiotic, germ free, or humanized.	Rodents have large cecum, are coprophagic, and rarely get diarrhea; colony-to-colony variation in microbiome.	Choice of species depends on question(s) asked.
	Pigs or dogs more like humans in GI tract anatomy and microbiology.	Expense, size, specialized training needed, and ethical issues.	
Animal diets	Chow-type diets inexpensive, provide all nutrients, high amount of fermentable substrate; open formula chow-type diets, such as NIH-31, do not vary over time.	Batch-to-batch variation, high polyphenol content alters microbiota; fiber content usually poorly characterized.	If chow-type diet, use open source formula.
	Purified diets allow complete control of all ingredients.	The prototypical diet, AIN-93, contains no fermentable substrate; leads to loss of bacterial diversity over multiple generations (39).	Purified diet is preferred for nutritional control but requires some fermentable substrate (soluble fiber and/or resistant starch).
	Drinking water can be standardized for pH, method of disinfection, etc.	Acidified water commonly used to control bacterial infections but leads to major changes in microbiota (44).	Describe this in Methods section.
In vitro systems	Operating conditions can be standardized, minimal ethics issues, easy sampling, and absence of host factors.	Choice of "best" inoculum, nature of nutrient supply, emulation of mucosal environment, high water flow; lack of donor diet history.	Describe donor health, diet, medication; single donor better than two, since one microbiota outcompetes the other.
Human studies	Nothing can replace controlled feeding studies in target population.	Poor adherence to specified diets; relatively inaccurate recording of dietary intake and composition; cross-sectional studies cannot prove causality.	Provide foods from metabolic kitchen; weigh uneaten food; alternatively, subjects need to record everything eaten.
	Wide variation in species and metabolites.	No agreement on what constitutes healthy microbiota; need a biomarker of dietary fiber intake; sampling of feces does not accurately reflect longitudinal or horizontal environments of the colon.	None yet
	Diets can be tightly controlled.	Difficult to feed diets low in polyphenols; not clear if fiber in diet should be based on dose, energy intake, or other factors; little information on how transit time affects microbiota.	Provide full detail in supplemental Methods so studies can be replicated or analyzed in a systematic review.

AIN-93, American Institute of Nutrition; GI, gastrointestinal; NIH, National Institutes of Health.

humans and even in vitro studies so that other researchers can reproduce the work. In fact, that approach is the way graduate students were taught to write papers decades ago. With the trend toward shorter Methods sections or placement of them in smaller font at the end of an article, it appears that some have devalued the importance of that section of a research manuscript that allows reproducibility, one of the fundamental tenets of the scientific method. Almost all journals are now online and offer online supplemental materials as an option; therefore, it behooves researchers to report even more detail about diet than would have been possible if an article were only in print, since there are few limitations on length of online materials. For example, a human study could include a spreadsheet with every food purchased for a controlled feeding study, including brand names of processed foods or the specification in as much detail as possible for fruits, vegetables, and grains, along with amounts consumed. This is prohibitive for observational studies that generally use food-frequency questionnaires or multiple dietary recalls, but efforts are underway to replace the older, recall-based methods with technologic approaches that reduce or avoid bias. More human studies are also needed to understand better how mixtures of fermentable carbohydrate affect the microbiota and to account for marked interindividual variation in response. We need data to determine if, for example, the Bristol Stool Scale can be used as a simple means of comparing dietary effects on fecal characteristics—and presumably, the microbiota to some extent—across studies. Basic information is not available on matters, such as whether the total number of bacteria is important; since dietary fiber contributes to increased fecal mass, and feces are ~50% bacteria, it is logical to assume that this information would be useful in understanding dietary modulation of the gut microbiota.

Animal studies need to provide more detail about the type of fiber in the diet. Even in the case of "cellulose," there are approximately two dozen commercially available types that differ in particle size, water-holding capacity, and potential for fermentation. A manufacturer may be consistent in use of one type of cellulose for animal diets, but there is no guarantee of that, particularly if suppliers change what is available over time, which is common in the food industry. If a soluble dietary fiber, such as pectin, is included in a diet, then the percent added to the diet, its viscosity, and its degree of methylation are important variables that potentially affect the microbiota differentially. Among the microbial metabolites studied, secondary bile acids and SCFAs have received the most attention; the latter accounts for 10% of the energy used by humans, but it is unknown if SCFAs are used with the same metabolic efficiency as macronutrients for the host and to what extent other bacteria use them for metabolic purposes. The pharmaceutical industry has embraced individuality in their studies, and it is likely that the same degree of uniqueness holds for studies of the diet and microbiome—not to the level of everyone being unique, but some limited number of metabotypes are likely to be identified, and the same holds for the intestinal microbiota. The field needs to move from associations to causality, and that will be catalyzed by the knowledge of, in as much detail as possible, what is ingested, how it is metabolized, and what health consequences derive from those processes.

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E.A.-V. is cofounder and Chief Scientific Officer of NuBiyota LLC, a company focused on commercializing microbial ecosystem therapeutics. B.R.H. is a partner in startup companies Nutrabiotix Inc. and Clostrabio LLC. D.M.K. is a consultant to Dyets, Inc., a manufacturer of laboratory animal diets. A.M. is a consultant to Danone Nutricia Research. B.O.S. serves on advisory boards for Monsanto and McCormick Spices. J. L. Slavin serves on advisory boards for Tate & Lyle, Kerry Ingredients, Atkins Nutritionals, and the Midwest Dairy Association. G.D.W. serves on scientific advisory boards for Janssen and Chr. Hansen and receives research funding from Nestle, Seres Therapeutics, Takeda, Intercept Pharmaceuticals, and Mead Johnson. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS

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