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Gut Microbiota in Health and Disease

INNA SEKIROV, SHANNON L. RUSSELL, L. CAETANO M. ANTUNES, AND B. BRETT FINLAY

Michael Smith Laboratories, Department of Microbiology and Immunology, and Department of Biochemistry and Molecular Biology, The University of British Columbia, Vancouver, British Columbia, Canada

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Sekirov I, Russell SL, Antunes LCM, Finlay BB. Gut Microbiota in Health and Disease. *Physiol Rev* 90: 859–904, 2010; doi:10.1152/physrev.00045.2009.—Gut microbiota is an assortment of microorganisms inhabiting the length and width of the mammalian gastrointestinal tract. The composition of this microbial community is host specific, evolving throughout an individual's lifetime and susceptible to both exogenous and endogenous modifications. Recent renewed interest in the structure and function of this "organ" has illuminated its central position in health and disease. The microbiota is intimately involved in numerous aspects of normal host physiology, from nutritional status to behavior and stress response. Additionally, they can be a central or a contributing cause of many diseases,

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affecting both near and far organ systems. The overall balance in the composition of the gut microbial community, as well as the presence or absence of key species capable of effecting specific responses, is important in ensuring homeostasis or lack thereof at the intestinal mucosa and beyond. The mechanisms through which microbiota exerts its beneficial or detrimental influences remain largely undefined, but include elaboration of signaling molecules and recognition of bacterial epitopes by both intestinal epithelial and mucosal immune cells. The advances in modeling and analysis of gut microbiota will further our knowledge of their role in health and disease, allowing customization of existing and future therapeutic and prophylactic modalities.

I. PREFACE

Hippocrates has been quoted as saying "death sits in the bowels" and "bad digestion is the root of all evil" in 400 B.C. (105), showing that the importance of the intestines in human health has been long recognized. In the past several decades, most research on the impact of bacteria in the intestinal environment has focused on gastrointestinal pathogens and the way they cause disease. However, there has recently been a considerable increase in the study of the effect that commensal microbes exert on the mammalian gut (Fig. 1). In this review, we revisit the current knowledge of the role played by the gastrointestinal microbiota in human health and disease. We describe the state-of-the-art techniques used to study the gastrointestinal microbiota and also present challenging questions to be addressed in the future of microbiota research.

II. OVERVIEW OF THE MAMMALIAN GUT MICROBIOTA

A. Humans as Microbial Depots

Virtually all multicellular organisms live in close association with surrounding microbes, and humans are no exception. The human body is inhabited by a vast number of bacteria, archaea, viruses, and unicellular eukaryotes. The collection of microorganisms that live in peaceful coexistence with their hosts has been referred to as the microbiota, microflora, or normal flora (154, 207, 210). The composition and roles of the bacteria that are part of this community have been intensely studied in the past few years. However, the roles of viruses, archaea, and unicellular eukaryotes that inhabit the mammalian body are less well known. It is estimated that the human microbiota contains as many as 10^{14} bacterial cells, a number that is 10 times greater than the number of human cells present in our bodies (162, 264, 334). The microbiota colonizes virtually every surface of the human body that is exposed to the external environment. Microbes flourish on our skin and in the genitourinary, gastrointestinal, and respiratory tracts (43, 126, 210, 323). By far the most heavily colonized organ is the gastrointestinal tract (GIT); the colon alone is estimated to contain over 70% of all the microbes in the human body (162, 334). The human gut has an estimated surface area of a tennis court (200 m^2) (85) and, as such a large organ, represents a major surface for microbial colonization. Additionally, the GIT is rich in molecules that can be used as nutrients by microbes, making it a preferred site for colonization.

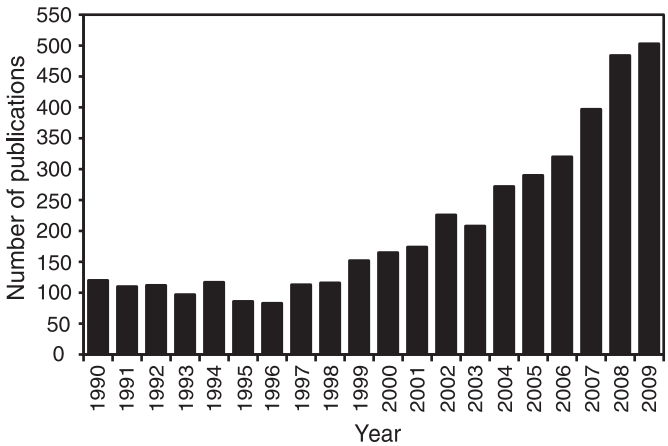


FIG. 1. Number of publications related to the intestinal microbiota in the last two decades, per year. Data were obtained by searching Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/) with the following terms: intestinal microbiota, gut microbiota, intestinal flora, gut flora, intestinal microflora, and gut microflora.

B. Who Are They?

The majority of the gut microbiota is composed of strict anaerobes, which dominate the facultative anaerobes and aerobes by two to three orders of magnitude (96, 104, 263). Although there have been over 50 bacterial phyla described to date (268), the human gut microbiota is dominated by only 2 of them: the Bacteroidetes and the Firmicutes, whereas Proteobacteria, Verrucomicrobia, Actinobacteria, Fusobacteria, and Cyanobacteria are present in minor proportions (64) (Fig. 2, *A* and *B*). Estimates of the number of bacterial species present in the human gut vary widely between different studies, but it has been generally accepted that it contains ~500 to 1,000 species (341). Nevertheless, a recent analysis involving multiple subjects has suggested that the collective human gut microbiota is composed of over 35,000 bacterial species (76).

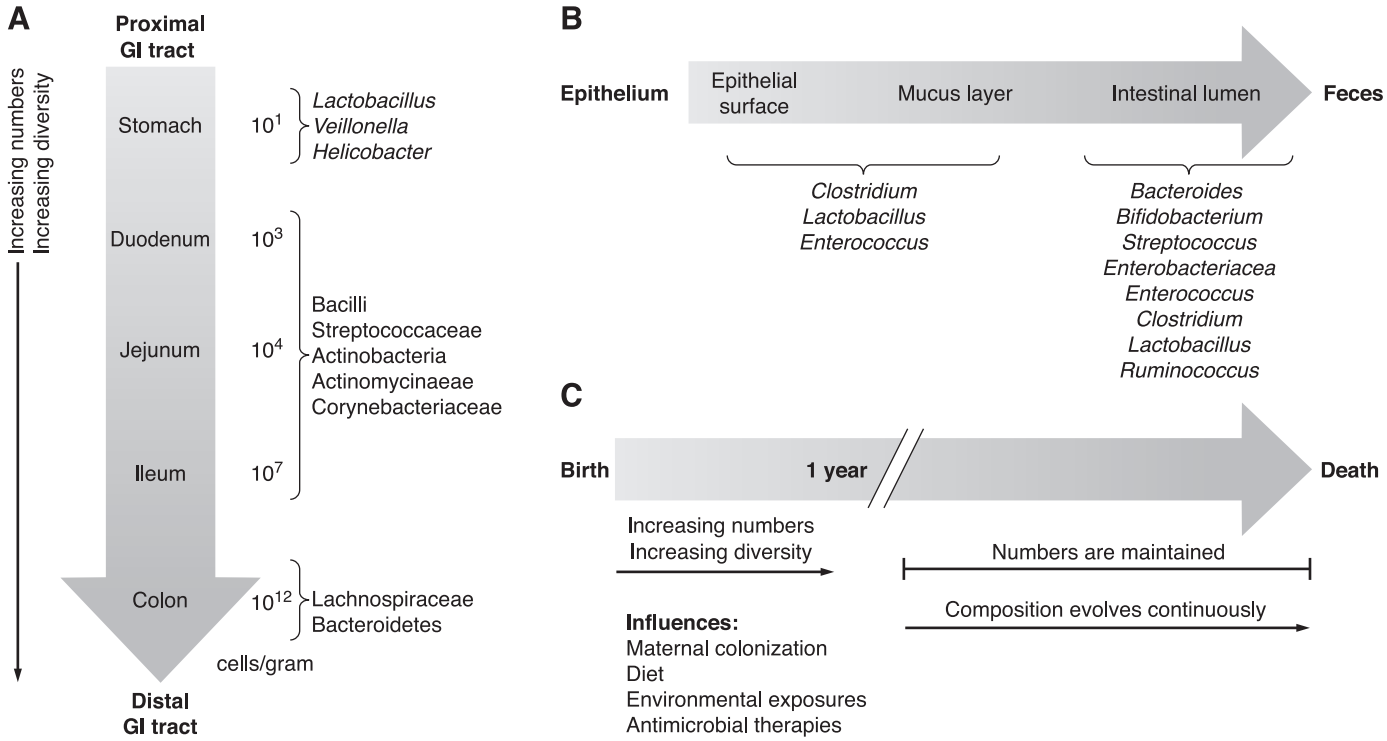


FIG. 2. Spatial and temporal aspects of intestinal microbiota composition. *A*: variations in microbial numbers and composition across the length of the gastrointestinal tract. *B*: longitudinal variations in microbial composition in the intestine. *C*: temporal aspects of microbiota establishment and maintenance and factors influencing microbial composition.

C. Where Are They?

The intestinal microbiota is not homogeneous. The number of bacterial cells present in the mammalian gut shows a continuum that goes from 10^1 to 10^3 bacteria per gram of contents in the stomach and duodenum, progressing to 10^4 to 10^7 bacteria per gram in the jejunum and ileum and culminating in 10^{11} to 10^{12} cells per gram in the colon (220) (Fig. 2*A*). Additionally, the microbial composition varies between these sites. Frank et al. (76) have reported that different bacterial groups are enriched at different sites when comparing biopsy samples of the small intestine and colon from healthy individuals. Samples from the small intestine were enriched for the Bacilli class of the Firmicutes and Actinobacteria. On the other hand, Bacteroidetes and the Lachnospiraceae family of the Firmicutes were more prevalent in colonic samples (76). In addition to the longitudinal heterogeneity displayed by the intestinal microbiota, there is also a great deal of latitudinal variation in the microbiota composition (Fig. 2*B*). The intestinal epithelium is separated from the lumen by a thick and physicochemically complex mucus layer. The microbiota present in the intestinal lumen differs significantly from the microbiota attached and embedded in this mucus layer as well as the microbiota present in the immediate proximity of the epithelium. Swidsinski et al. (303) have found that many bacterial species present in the intestinal lumen did not access the mucus layer and epithelial crypts. For instance, *Bacteroides*, *Bifidobacterium*, *Streptococcus*, members of Enterobacteriacea, *Enterococcus*, *Clostridium*, *Lactobacillus*, and *Ruminococcus* were all found in feces, whereas only *Clostridium*, *Lactobacillus*, and *Enterococcus* were detected in the mucus layer and epithelial crypts of the small intestine (303).

D. Where Do They Come From?

Colonization of the human gut with microbes begins immediately at birth (Fig. 2*C*). Upon passage through the birth canal, infants are exposed to a complex microbial population (245). Evidence that the immediate contact with microbes during birth can affect the development of the intestinal microbiota comes from the fact that the intestinal microbiota of infants and the vaginal microbiota of their mothers show similarities (187). Additionally, infants delivered through cesarean section have different microbial compositions compared with vaginally delivered infants (128). After the initial establishment of the intestinal microbiota and during the first year of life, the microbial composition of the mammalian intestine is relatively simple and varies widely between different individuals and also with time (179, 187). However, after 1 yr of age, the intestinal microbiota of children starts to

resemble that of a young adult and stabilizes (179, 187) (Fig. 2*C*). It is presumed that this initial colonization is involved in shaping the composition of the gut microbiota through adulthood. For instance, a few studies have shown that kinship seems to be involved in determining the composition of the gut microbiota. Ley et al. (161) have shown that, in mice, the microbiota of offspring is closely related to that of their mothers. Additionally, it has been shown that the microbiota of adult monozygotic and dizygotic twins were equally similar to that of their siblings, suggesting that the colonization by the microbiota from a shared mother was more decisive in determining their adult microbiota than their genetic makeup (350). Although these studies point to the idea that parental inoculation is a major factor in shaping our gut microbial community, there are several confounding factors that prohibit a definite conclusion on this subject. For example, it is difficult to take into account differences in diet when human studies are performed. On the other hand, mouse studies are performed in highly controlled environments, where exposure to microbes from sources other than littermates and parents is limited. Therefore, further investigation is needed to decisively establish the role of parental inoculation in determining the composition of the adult gut microbiota.

E. How Are They Selected?

Besides the mother's microbiota composition, many other factors have been found to contribute to the microbial makeup of the mammalian GIT (Fig. 2*C*). Several studies have shown that host genetics can impact the microbial composition of the gut. For instance, the proportions of the major bacterial groups in the murine intestine are altered in genetically obese mice, compared with their genetically lean siblings (161). Also, mice containing a mutation in the major component of the high-density lipoprotein (apolipoprotein a-I) have an altered microbiota (347). Although these studies suggest that host genetics can have an impact on the gut microbiota, it should be noted that such effects are likely to be indirect, working through effects on general host metabolism.

Studies on obesity have also revealed that diet can affect gut microbial composition. Consumption of a prototypic western diet that induced weight gain significantly altered the microbial composition of the murine gut (311). Further dietary manipulations that limited weight gain were able to reverse the effects of diet-induced obesity on the microbiota.

Given the plethora of factors that can affect microbial composition in the human gut, it is perhaps surprising that the composition of the human microbiota is fairly stable at the phylum level. The major groups that dominate the human intestine are conserved between all individuals, although the proportions of these groups can vary. However, when genera and species composition within the human gut is analyzed, differences occur. Within phyla, the interindividual variation of species composition is considerably high (64, 89). This suggests that although there is a selective pressure for the maintenance of certain microbial groups (phyla) in the microbiota, the functional redundancy within those groups allows for variations in the composition of the microbiota between individuals without compromising the maintenance of proper function. However, this hypothesis remains to be experimentally tested.

III. MICROBIOTA IN HEALTH: COMBINE AND CONQUER

Several lines of evidence point towards a possible coevolution of the host and its indigenous microbiota: it has been shown that transplantation of microbial communities between different host species results in the transplanted community morphing to resemble the native microbiota of the recipient host (242), and that gut microbiota species exhibit a high level of adaptation to their habitat and to each other, presenting a case of "microevolution" that paralleled the evolution of our species on the large scale (257, 342). Moreover, the host has evolved intricate mechanisms that allow local control of the resident microbiota without the induction of concurrent damaging systemic immune responses (181).

This adaptation is not surprising when considering that different bacterial groups and species have been implicated in various aspects of normal intestinal development and function of their host (Fig. 3). In recent years, we have seen a tremendous increase in gut microbiota-related research, with important advances made towards establishing the identity of specific microbes/microbial groups or microbial molecules contributing to various aspects of host physiology. Concurrently, host factors involved in various aspects of development and maturation targeted by the microbiota have been identified. However, a large proportion of research aimed at identifying particular microbiota contributors to host health was done in ex-germ-free (GF) animals mono- or poly-associated with different bacterial species representative of dominant microbiota phyla (e.g., *Bacteroides thetaiotaomicron*, *Bacteroides fragilis*, *Lactobacillus spp.*) or stimulated with particular microbial components [e.g., lipopolysaccharide (LPS) and polysaccharide A (PSA)]. Thus any discovered contribution of these particular microbial species or molecules to a distinct host structure/function points to their ability to provide the said contribution, but not to the fact that they are the primary microbe/molecule responsible for it in a host associated with a complete microbial community. Additionally, as

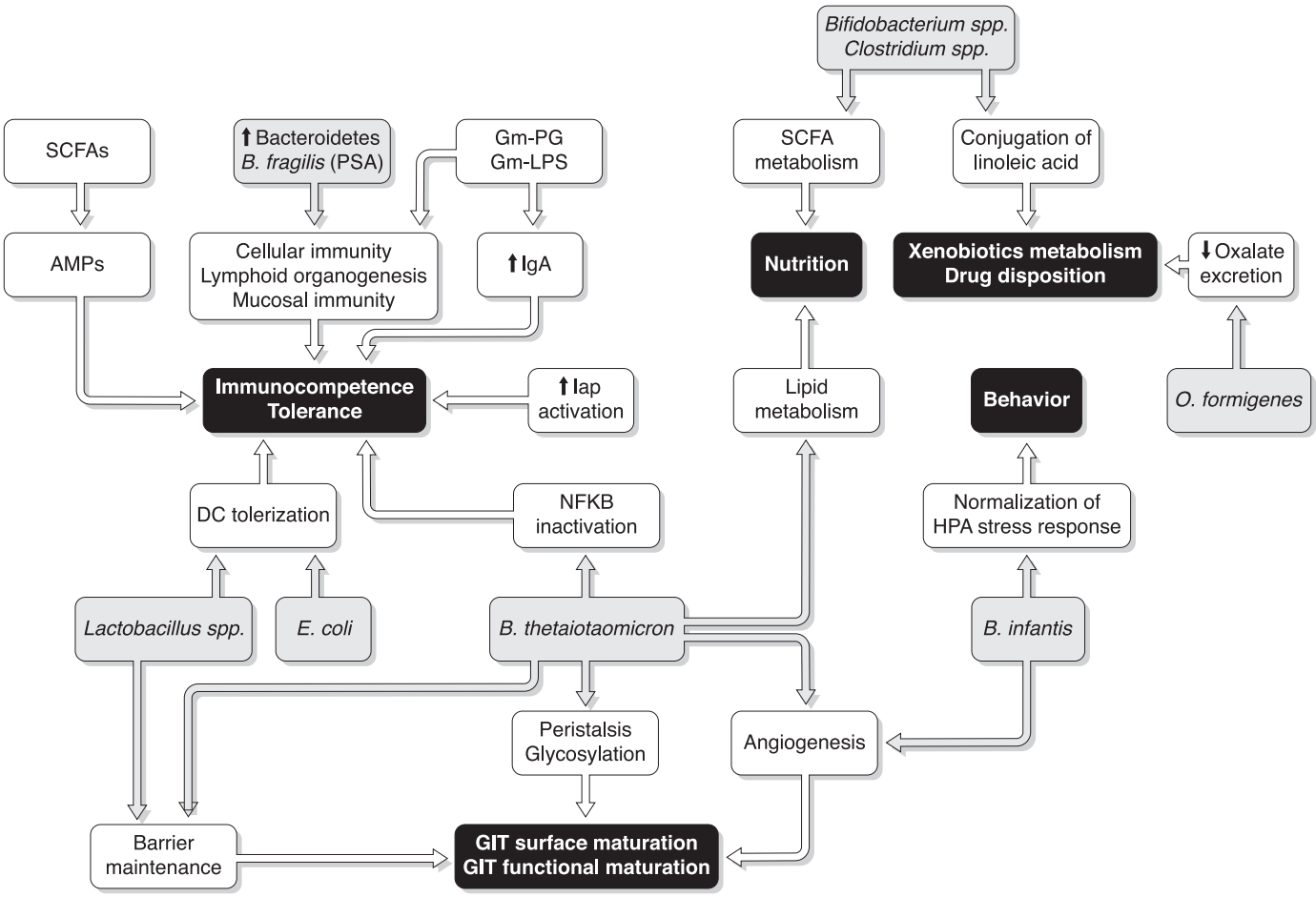


FIG. 3. The complex web of gut microbiota contributions to host physiology. Different gut microbiota components can affect many aspects of normal host development, while the microbiota as a whole often exhibits functional redundancy. In gray are shown members of the microbiota, with their components or products of their metabolism. In white are shown their effects on the host at the cellular or organ level. Black ellipses represent the affected host phenotypes. Only some examples of microbial members/components contributing to any given phenotype are shown. AMP, antimicrobial peptides; DC, dendritic cells; Gm−, Gram negative; HPA, hypothalamus-pituitary-adrenal; Iap, intestinal alkaline phosphatase; PG, peptidoglycan; PSA, polysaccharide A.

current culturing techniques limit our ability to isolate strictly anaerobic microbiota members or members with complex nutrient requirements and mutualistic dependence on other microbial gut inhabitants (62), the research on the contribution of specific gut microbes to various physiological processes is limited to studying a small number of currently isolated and culturable microorganisms. However, improvements to available culturing techniques (62) and enhanced understanding of microbial metabolism gained from culture-independent studies hold promise to greatly expand this field of research.

A. Immunomodulation

The importance of the gut microbiota in the development of both the intestinal mucosal and systemic immune systems can be readily appreciated from studies of GF (microbiota lacking) animals. GF animals contain abnormal numbers of several immune cell types and immune cell products, as well as have deficits in local and systemic lymphoid structures. Spleens and lymph nodes of GF mice are poorly formed. GF mice also have hypoplastic Peyer's patches (PP) (180) and a decreased number of mature isolated lymphoid follicles (27). The number of their IgA-producing plasma cells is reduced, as are the levels of secreted immunoglobulins (both IgA and IgG) (180). They also exhibit irregularities in cytokine levels and profiles (220) and are impaired in the generation of oral tolerance (132).

The central role of gut microbiota in the development of mucosal immunity is not surprising, considering that the intestinal mucosa represents the largest surface area in contact with the antigens of the external environment and that the dense carpet of the gut microbiota overlying the mucosa normally accounts for the largest proportion of the antigens presented to the resident immune cells

and those stimulating the pattern recognition receptors [such as the TLRs and NOD-like receptors (NLRs)] of the intestinal epithelial cells (238). A detailed overview of the intestinal mucosal immunity can be found elsewhere (110, 194). Briefly, it is composed of the gut-associated lymphoid tissue (GALT), such as the PP and small intestinal lymphoid tissue (SILT) in the small intestine, lymphoid aggregates in the large intestine, and diffusely spread immune cells in the lamina propria of the GIT. These immune cells are in contact with the rest of the immune system via local mesenteric lymph nodes (MLN). In addition to the immune cells, the intestinal epithelium also plays a role in the generation of immune responses through sampling of foreign antigens via TLRs and NLRs (238).

The mucosal immune system needs to fulfill two, sometimes seemingly conflicting, functions. It needs to be tolerant of the overlying microbiota to prevent the induction of an excessive and detrimental systemic immune response, yet it needs to be able to control the gut microbiota to prevent its overgrowth and translocation to systemic sites. Gut microbiota is intricately involved in achieving these objectives of the GIT mucosal immune system.

1. *Mucosal/systemic immunity maturation and development*

A major immune deficiency exhibited by GF animals is the lack of expansion of CD4+ T-cell populations. This deficiency can be completely reversed by treatment of GF mice with PSA of *Bacteroides fragilis* (197). Mazmanian et al. (197), in an elegant series of experiments, have shown that either mono-association of GF mice with *B. fragilis* or oral treatment with its capsular antigen PSA induces proliferation of CD4+ T cells, as well as restores the development of lymphocytes-containing spleen white pulp. Recognition of PSA by dendritic cells (DCs) with subsequent presentation to immature T lymphocytes in MLNs was required to promote the expansion. GF animals exhibit systemic skewing towards a Th2 cytokine profile, a phenotype that was shown to be reversed by PSA treatment, in a process requiring signaling through the interleukin (IL)-12/Stat4 pathway (197). Thus exposure to a single structural component of a common gut microbiota member promotes host immune maturation both locally and systemically, at the molecular, cellular, and organ levels.

While *B. fragilis* PSA appears to have a pan-systemic effect on its host's immunological development, additional gut microbiota constituents and their components have been shown to have immunomodulatory capacity, highlighting the overlapping, and possibly additive or synergistic, functions of the members of the gut microbial community. For instance, various *Lactobacilli spp.* have been shown to differentially regulate DCs, with consequent influence on the Th1/Th2/Th3 cytokine balance at the intestinal mucosa (44), as well as on the activation of natural killer (NK) cells (72). Additionally, peptidoglycan of Gram-negative bacteria induces formation of isolated lymphoid follicles (ILF) via NOD1 (an NLR) signaling. Following recognition of microbiota through TLRs, these ILF matured into B-cell clusters (27).

A complex microbial community containing a significant proportion of bacteria from the Bacteroidetes phylum was shown to be required for the differentiation of inflammatory Th17 cells (133). Interestingly, the colonization of GF mice with altered Schaedler flora (ASF) was insufficient to promote differentiation of Th17 cells, despite the fact that ASF includes a number of bacteria from the Bacteroidetes phylum (59). This finding highlights the complexity of interactions between the host and the microbiota and within the microbiota community, indicating that cooperation between microbiota members may be required to promote normal host development. In view of this, the finding by Atarashi et al. (9), that administration of ATP (which is found in high concentrations in the GIT of SPF, but not GF mice) was sufficient to trigger differentiation of Th17 cells in GF mice, is all the more intriguing. This raises questions about the metabolic capabilities of different members of the gut microbiota and lends indirect evidence to their metabolic interdependence.

2. *Tolerance at the GIT mucosa*

The GIT needs to coexist with the dense carpet of bacteria overlying it without an induction of excessive detrimental immune activation both locally and systemically. Prevention of excessive immune response to the myriad of bacteria from the gut microbiota can be achieved either through physical separation of bacteria and host cells, modifications of antigenic moieties of the microbiota to render them less immunogenic, or modulation of localized host immune response towards tolerance.

Resident immune cells of the GIT often have a phenotype distinct from cells of the same lineage found systemically. For instance, DCs found in the intestinal mucosa preferentially induce differentiation of resident T cells into Th2 (134) and Treg (144) subsets, consequently promoting a more tolerogenic state in the GIT. In a series of in vitro experiments, DCs were conditioned towards this tolerogenic phenotype by intestinal epithelial cells (IEC) stimulated with various gut microbiota isolates, such as different *Lactobacillus spp.* and different *Escherichia coli* strains (346). The conditioning was dependent on microbiota-induced secretion of TSLP and transforming growth factor (TGF)-β by IECs (346). Interestingly, the Gram-positive *Lactobacilli* were more effective than the Gram-negative *E. coli* in conditioning the DCs towards a tolerogenic

phenotype, likely due to the greater abundance of *Lactobacilli* at the intestinal mucosa, as hypothesized by the authors of the study. Another effective mechanism of preventing colitogenic responses is employed by *B. thetaiotaomicron*, which prevents activation of the proinflammatory transcription factor NFκB by promoting nuclear export of a transcriptionally active NFκB subunit RelA in a PPARγ-dependent fashion (143). An alternate mechanism of preventing NFκB activation in response to the gut microbiota is through TLR compartmentalization. Lee et al. (159) have shown that while activation of basolaterally located TLR9 promotes NFκB activation, signaling originating from the apical surfaces (i.e., induced by normal gut microbiota) effectively prevents NFκB activation, promoting tolerance to the resident bacteria.

In addition to microbiota-mediated tolerogenic skewing of localized immune responses, the host can also decrease the proinflammatory potential of microbiota constituents. The presence of the gut microbiota exposes the host to a vast amount of LPS found on the outer membranes of Gram-negative bacteria. Systemic reactions to LPS lead to highly lethal septic shock (19), a very undesirable outcome of host-microbiota interactions. One way to avoid this disastrous scenario is to minimize the toxic potential of LPS, which can be done via dephosphorylation of the LPS endotoxin component through the action of alkaline phosphatases, specifically the intestinal alkaline phosphatase (Iap) (18). Bates et al. (18) have demonstrated that Iap activity in the GIT of zebrafish reduced MyD88- and tumor necrosis factor (TNF)-α-mediated recruitment of neutrophils to the intestinal epithelium, minimizing the inflammatory response to the gut microbiota and promoting tolerance. Iap activity in zebrafish GIT was induced via MyD88 signaling and was dependent on the presence of microbiota: it could be induced by mono-association with Gram-negative (Gm−) bacterial isolates (such as *Aeromonas* and *Pseudomonas*) or treatment with LPS. Association with Gram-positive (Gm+) bacterial isolates (such as *Streptococcus* and *Staphylococcus*) failed to promote Iap activity (18), demonstrating that at least some host responses to its colonizing microbes are group specific.

In addition to detoxification of LPS by Iap, IECs also acquire tolerance to endotoxin through downregulation of IRAK-1, which is essential for endotoxin signaling through TLR4 (174). This tolerance is acquired at birth, but only in vaginally delivered mice that were exposed to exogenous LPS during passage through the birth canal (174), again highlighting the active role of the microbiota in tolerogenic conditioning of mucosal immune responses at the GIT.

Another effective strategy of avoiding excessive immune activation at the intestinal mucosa is physical separation of the microbiota from the host mucosal immune system. Recently, Johansson et al. (136) have shown that the mucus layer overlying the colonic mucosa is effectively divided into two tiers, with the bottom tier being devoid of bacteria, and the more dynamic top tier being permeated by members of the gut microbiota.

3. *Control of the gut microbiota*

While healthy gut microbiota is essential to promote host health and well-being, overgrowth of the bacterial population results in a variety of detrimental conditions, and different strategies are employed by the host to prevent this outcome.

Plasma cells residing at the intestinal mucosa produce secretory IgA (sIgA) that coats the gut microbiota and allows local control of their numbers (181, 310). They are activated by resident DCs that sample the luminal bacteria, but are restricted in their migration to only as far as the local MLNs, so as to avoid induction of a systemic response to the gut microbiota (181). The presence of the gut microbiota is a prerequisite to activate gut DCs to induce maximal levels of IgA production, while treatment of GF mice with LPS augmented IgA production but to lower levels (195). Furthermore, *Bacteroides* (Gm− bacteria) were found to be more efficient in induction of sIgA than *Lactobacilli* (Gm+ bacteria) (343). Interestingly, although Gm− bacteria or their structural components were able to stimulate IgA production, the absence of intestinal IgA resulted in overgrowth of SFB, a group of Gm+ bacteria (300), suggesting that induction of sIgA might also be a form of competition between different microbiota members.

Two secretory IgA (sIgA) subclasses exist: sIgA1 (produced systemically and at mucosal surfaces) and sIgA2 (produced at mucosal surfaces). sIgA2 is more resistant to degradation by bacterial proteases than sIgA1 (202), so it is not surprising that it was found to be the main IgA subclass produced in the intestinal lamina propria (107). Production of a proliferation-inducing ligand (APRIL) by IECs activated via TLR-mediated sensing of bacteria and bacterial products was required to induce switching from sIgA1 to sIgA2 production (107). Both Gm+ and Gm− bacteria, as well as bacterial LPS and flagellin, were similarly effective in inducing APRIL production (107). Thus exposure of the gut mucosa to its resident microbiota not only promotes IgA secretion, but also ensures that the optimally stable IgA subclass is produced. It is also of interest to note that sIgA fulfills a dual function at the intestinal mucosa: in addition to preventing overgrowth of the gut microbiota, it also minimizes its interactions with the mucosal immune system, diminishing the host's reaction to its resident microbes (234).

sIgA is not the only host factor preventing the microbiota from breaching its luminal compartment: antimicrobial peptides (AMP) produced by the host also work to

this end (mechanisms of induction of AMP production are discussed in the following section). AMPs were found to have a different spatial distribution along the width of the GIT (204). Maximal antimicrobial activity was observed in the intestinal crypts, as well as in the mucus layer overlying the mucosa, whereas the inhibition of microbial growth in the lumen was markedly reduced (204). This demonstrates that AMPs are preserved close to the intestinal mucosa by the mucus layer, preventing the gut microbiota from breaching its luminal niche. At the same time, the diminished luminal antimicrobial activity allows local replication of the gut microbes while limiting their contact with the host epithelium.

B. Protection

Gut microbiota provides its host with a physical barrier to incoming pathogens by competitive exclusion, such as occupation of attachment sites, consumption of nutrient sources, and production of antimicrobial substances. It also stimulates the host to produce various antimicrobial compounds.

Numerous AMPs, such as defensins, cathelicidins, and C-type lectins, are produced in the mammalian GIT; they are a diverse group of compounds that act by disrupting surface structures of both commensal and pathogenic bacteria (118, 255). While one of the main functions of AMPs is the regulation of composition and numbers of the intestinal microbiota (255), the interactions of AMPs and microbiota are bidirectional, as various microbial species, as well as products of microbial metabolism, have been shown to stimulate production of different types of AMPs.

Paneth cells, a cell type of epithelial lineage found at the base of small intestinal crypts, express a variety of AMPs. This expression is directed by the presence of normal gut microbiota (39, 317). Interestingly, while the presence of the whole microbial community was necessary to promote full levels of AMP expression, somewhat lower levels of transcripts could be induced by the presence of single bacterial species, such as *B. thetaiotaomicron* (39, 120) and *L. innocua* (39) or stimulation with LPS (120, 317). It appeared that for induction to occur, the commensal bacteria had to be in close contact with the intestinal epithelium, as single microbial species were able to produce a much higher induction when administered to RAG1−/− mice (lacking secretory IgA that sequesters luminal bacteria) than to wild-type (WT) mice (39). The induction was mediated through TLR-MyD88 signaling (317).

In addition to microbial cells or microbial structural components, microbial metabolites also have the ability to induce AMP expression in vivo and in several cell lines. Short-chain fatty acids (SCFAs) and lithocholic acid were shown to induce expression of LL-37 cathelicidin (147, 267, 309). The induction involved the MEK/ERK pathway (267), AP-1 transcription factor, and histone acetylation (147).

Some AMPs (e.g., defensins) are initially produced in an inactive form (e.g., prodefensins), which needs to be proteolytically cleaved to be activated. Paneth cells produce matrilysin, a matrix metalloproteinase that activates defensins, and *B. thetaiotaomicron* colonization of GF mice was shown to induce matrilysin expression (172), demonstrating another aspect of microbiota-mediated induction of antimicrobial host defenses.

Thus it appears that the presence of commensal bacteria or their structural components, as well as the presence of products of bacterial metabolism have the capacity to induce the expression of AMPs and promote their activation, contributing to host protection against invading pathogens and preventing the overgrowth of the commensals themselves. Induction can be mediated through different signaling pathways, reflecting the different nature of the inductive stimuli.

The physical presence of the microbiota in the GIT also serves as a deterrent to pathogen colonization. A lot of studies, especially in the probiotics field of research, have contributed to the identification of different bacterial species with antagonistic activities against different pathogens, although the description of the exact mechanisms underlying this antagonism is often lacking (260).

Gm+ anaerobic fecal isolates were shown to have a greater inhibitory effect on the growth of enteric pathogens in vitro than Gm− anaerobic isolates (91). The antagonistic activity, however, was quite variable between isolates from different volunteers, as well as from different time points, highlighting the interindividual variations of gut microbiota and its propensity for dynamic fluctuations over time.

A number of commonly utilized probiotic strains prevent attachment and invasion of various bacterial pathogens. The Gm+ *Lactobacillus* and *Bifidobacterium*, facultatively and obligately anaerobic, respectively, were shown to prevent *Listeria* infection of cultured epithelial cells through both the elaboration of secreted compounds and modulation of the epithelial cells' immune response to *Listeria* (48). Compounds secreted by *Lactobacillus* were also shown to decrease in vivo colonization by pathogenic *E. coli* (201). Additionally, the presence of SFB on ileal mucosa was suggested to physically exclude *S. enteritidis* from its attachment sites (82), as well as to prevent colonization of rabbits by rabbit enteropathogenic *E. coli* (108).

Members of the *Lactobacillus* genus produce lactic acid which, in addition to providing an inhibitory environment to the growth of many bacteria, also potentiates the antimicrobial activity of host lysozyme by disrupting the bacterial outer membrane (4). Other microbiota isolates

also produce antimicrobial substances (53, 57, 92, 240, 247); their production is sometimes dependent on host factors, such as trypsin (53, 92, 240), demonstrating the adaptation of gut microbiota to its environment. These antimicrobial substances often tend to exhibit antimicrobial activity against bacterial groups similar to the producer, possibly in a strategy aimed at keeping potential competitors out of the producer's favored intestinal niches, but also benefiting the host along the way. However, some microbiota isolates, specifically of the *Lactobacillus spp.*, produce antimicrobial substances that are active against a wide range of enteropathogenic bacteria, both Gm− and Gm+ (166).

C. Structure and Function of the GIT

Newborns exit the intrauterine environment with a structurally and functionally immature GIT (325); its postnatal development is influenced by a number of factors, including exposure to a developing gut microbial community (254, 325). Comparisons of GF and SPF animals reveal the central role of gut microbiota in the structural and functional development of the GIT. GF ceca are greatly enlarged, often leading to reproductive and functional gastrointestinal disorders (338). The GF gut presents an increased enterochromaffin cell area (220), while the intestinal surface area is reduced (94). Smaller villus thickness, resulting from reduced cell regeneration (14) and increased cell cycle time (5), or from reduced leukocyte infiltration into the lamina propria, might account for the smaller gut surface area. Many aspects of the GF gut function are also compromised: a severe reduction in the villous capillary network of GF mice (288) has many potential implications for nutrient absorption. There is an impairment in the peristaltic activity of the GF GIT (127). Additionally, GF animals have abnormal cholesterol and bile acid metabolism: GF rats exhibit defective bile acid deconjugation (182) and a reduced rate of systemic cholesterol metabolism (339), while GF mice show increased hepatic cholesterol accumulation (99).

Recent research has examined several aspects of the host-microbiota interactions that promote functional and structural maturation of the GIT. To reach maturity, the GIT needs to develop efficient peristaltic motility, as well as a sufficient surface area and blood supply for nutrient acquisition. It needs to contain adequate attachment sites that can support the resident bacterial community, while being resistant to systemic translocation of food- and microbiota-derived foreign antigens. Ultimately, the GIT needs to be able to maintain its homeostasis and regenerate following an injury.

1. *Peristalsis and surface maturation*

While previously discussed evidence from GF animals implicates the gut flora in postnatal growth of intestinal surface area, the identities of microbes/microbial molecules responsible for this development, as well as the signaling pathways through which it is stimulated, remain elusive. However, some elegant studies have provided interesting insights into the role of microbiota in the development of intestinal microvasculature. A number of microbiota members have been shown to induce transcription of angiogenin-3, a protein with angiogenic activity (121). It is intriguing that while colonization of ex-GF mice with *Bacteroides thetaiotaomicron* (a prominent member of postweaning gut microbiota) resulted in the same transcriptional levels of angiogenin-3 as those observed in SPF mice, colonization with *Bifidobacterium infantis* (a pioneer of the GIT commonly found in newborn microbiota) resulted in lower transcription of angiogenin-3. This finding suggests that temporal maturation of the gut microbiota is at least partially responsible for sequential maturation of the GIT. *B. thetaiotaomicron*-induced angiogenesis was shown to depend on signaling via Paneth cells (288). Colonization of ex-GF mice with *B. thetaiotaomicron* was also shown to influence transcription of various host factors involved in function of the enteric nervous system, suggesting that it can modulate postnatal development of peristalsis (121).

Carbohydrate moieties frequently serve as microbial attachment sites or nutrient sources, making mucosal glycosylation patterns an important factor in colonization of GIT by the gut microbiota (230). While the host has innate mechanisms in place to regulate the spatial and cell-specific distribution of glycan expression, indicating that the host is armed with a full arsenal of glycosyltransferases necessary for glycosylation processes, the glycosylation patterns are further modified by the presence of the gut microbiota (77). Microbiota-induced modifications happen at both the cellular (quantitative and qualitative differences in surface glycan expression on different cell types) and the subcellular (modifications of trafficking of glycan-bearing structures) levels, and it has been shown that *B. thetaiotaomicron* secretes a signaling molecule that induces the host to express fucose on cell surface glycoconjugates, which can then be released and consumed by *B. thetaiotaomicron* (119). This finding demonstrates that the gut microbiota is able to generate a suitable physiological niche by modulating the intestinal glycocalyx structure.

2. *Barrier fortifications and regenerative capacity*

Preservation of homeostasis at the intestinal mucosa should be in the gut microbiota's best interest, as it provides a convenient long-term habitat. It should not be surprising then that various microbiota members contribute to the maintenance of intestinal epithelium barrier integrity through maintenance of cell-to-cell junctions and promotion of epithelial repair following injury.

B. thetaiotaomicron has been shown to induce expression of sprr2a, important in desmosome maintenance (121). The expression was increased at the epithelial villus, suggesting its role in barrier maintenance. Several probiotic strains of *Lactobacillus* have been shown to contribute to the maintenance of tight junctions in intestinal epithelia, providing protective effect in the face of pathogen assault or intestinal injury (176). Furthermore, signaling via TLR2, which in vivo is principally stimulated by microbial cell wall peptidoglycan, was shown to promote the integrity of the intestinal epithelium through maintenance of tight junctions and decreased apoptosis (38). Microbiota signaling through mucosal TLRs was also shown to be required for maintenance of intestinal epithelial homeostasis and repair following intestinal injury (239).

D. Outside of the GIT

The importance of a healthy gut microbiota is not confined to the GIT itself. A number of extraintestinal processes and organ systems are dysregulated in GF animals, highlighting the contributions of the indigenous gut microbes to their development and maintenance. A GF state results in several impairments to the cardiovascular system. The cardiac output of GF animals is reduced compared with the SPF state (95), while the mesenteric vasculature is hypotonic with a reduced reactivity to vasoactive substances (12). GF mice under nutritional deprivation are unable to utilize the same energy sources for cardiac metabolism as SPF mice and exhibit lower myocardial weight (50).

Additionally, the development of the nervous systems is affected in the GF state, with abnormalities ranging from the dysregulation of hypothalamic-pituitary-adrenal (HPA) axis (296, 297) to decreased perception of inflammatory pain (7). Recently, there has been renewed interest in the role of microbiota in the development of both central and peripheral neural processes. These interactions, termed the "brain-gut-enteric microbiota axis" (246), are often bidirectional, with potential implications of disruption of this axis spanning from abnormal neurogenic stimulation of the enteric nervous system (the irritable bowel syndrome or IBS) to depression.

Gut microbiota has been shown to influence the development of the (HPA) axis, specifically its "set point," influencing the host response to stress (296, 297). Monoassociation of ex-GF mice with *Bifidobacterium infantis* was sufficient to normalize the stress response, but only if reconstitution occurred in young animals (297), demonstrating that a limited window of opportunity may exist for normal development of some of the host processes. The gut microbiota was also linked to the control of levels of various signaling molecules (such as brain-derived neurotrophic factor, norepinephrine, and tryptophan) in different areas of central nervous system, both in the cortex and the brain stem (74). These observations prompted many to hypothesize on the role of the microbiota in the regulation of mood and behavior, and their contribution to the pathophysiology of mood disorders (47, 74, 211, 246).

The brain has the ability to "sense" gut bacteria; introduction of pathogenic bacteria into ceca of rodent models activated several brain stem nuclei (paraventricular, supraoptic, parabrachial and tractus solitarius nuclei), indicating that bacterial signals are relayed to the central nervous system (47). The afferent vagus nerve, which originates in the brain stem and innervates abdominal organs relaying visceral sensory information to the brain, was implicated as the possible route of bacterial signal transmission (47).

Another behavioral aspect that was recently suggested to be partially mediated by the gut microbiota is appetite control (68, 69). Autoantibodies against several key appetite-regulating hormones have been found in both healthy human subjects and rodent models with many of the targeted epitopes showing sequence homology to members of the gut microbiota. As levels of several of these autoantibodies were altered in GF rats, it was hypothesized that microbiota composition plays a role in regulating the levels and types of autoantibodies targeting the appetite-regulating hormones, and consequently regulates appetite control-related aspects of behavior. Along similar lines, the gut microbiota was hypothesized to play a role in the pathophysiology of eating disorders.

E. Nutrition and Metabolism

The genetic information contained by the myriad of gut microbes encodes for a far more versatile metabolome than that found in the human genome (89). The study of the sum of our own metabolic abilities and those of our gut microbiota constituents is referred to as metabonomics (discussed in more detail in section VII*G3*), and our own contribution to many of the metabolic processes essential for our homeostasis is remarkably small compared with the share provided by the microbiota. A large proportion of the microbiota metabolic processes beneficial to the host is involved in either nutrient acquisition or xenobiotic processing.

1. Microbiota and body weight

The observation that GF animals require a significantly higher caloric intake to maintain the same body weight as SPF animals prompted investigations into the mechanisms through which gut microbiota maximizes caloric availability of ingested nutrients. These mechanisms generally fall into one of two categories: extraction

of additional calories from otherwise indigestible oligosaccharides and promotion of nutrient uptake and utilization by modulation of absorptive capacity of the intestinal epithelium and ultimate nutrient metabolism.

Many bacterial species have been implicated in metabolism of dietary fiber to SCFA, accounting for a significant part of the human energy source (178, 262). Production of some of these SCFA, such as butyrate, is not only important as an energy source for the host, but also prevents the accumulation of potentially toxic (319) metabolic by-products, such as D-lactate (26, 63).

In addition to being able to break down indigestible polysaccharides to absorbable monosaccharides, the intestinal microbiota also modulates the uptake and deposition of dietary lipids. Gut microbiota was shown to suppress the inhibition of lipoprotein lipase (LPL); increased LPL activity in adipose tissues promoted increased fatty acid uptake into adipocytes (10). Additionally, mono-association of ex-GF mice with *B. thetaiotaomicron* was shown to upregulate the expression of colipase, a cofactor needed by pancreatic lipase for efficient hydrolysis of dietary lipids (121). An upregulation of a Na^+/glucose cotransporter at the intestinal epithelium, with a likely increase in glucose uptake, was also observed (121). Gpr41, a G protein-coupled receptor that binds SCFAs, and PYY, an enteroendocrine hormone, were shown to be involved in microbiota-dependent regulation of host energy balance (258).

Nutrient metabolism by resident microbes is not carried out strictly for the host's benefit; part of the energy extracted from luminal nutrients is designated for the microbiota itself, to maintain its numbers and fitness. It has been shown that members of the gut microbiota are able to adapt their metabolism to the conditions of the intestine, responding to substrate availability. Intestinal *E. coli* expressed a different set of proteins involved in nutrient utilization (chiefly those involved in amino acid and carbohydrate transport and metabolism) than *E. coli* cultured in vitro under anaerobic conditions (6). Even more notably, significant differences were observed between fecal and cecal *E. coli*, possibly due to the different nutrient availability in the lumen versus closer to the intestinal mucosa. *B. thetaiotaomicron* was also shown to vary its metabolism based on intestinal nutrient availability (193), utilizing dietary carbohydrates during periods of their abundance, but turning to digest the host's mucus layer under carbohydrate depletion conditions (280). In addition, resident bacteria were shown to adapt to the presence of other microbiota members, striving to maximize their fitness in the GIT by selective nutrient utilization (186). Regulation of gut microbiota metabolism was shown to happen both at the nucleic acid (186) and protein (6, 186) levels.

2. *Microbiota effects on drugs*

Variations in the gut microbiome have been linked to the modulation of human metabolic phenotypes, with different members of the gut microbiota exerting various degrees of influence on the presence of different metabolites (165). These interindividual and interpopulation differences in gut microbiomes with consequent differences in metabonomes have been suggested to account for different toxicities of commonly used therapeutics in different geographic and cultural populations (212). Our emerging appreciation of the gut microbiota's contribution to the metabolism of xenobiotic compounds, including therapeutics, will influence future toxicological studies in the pharmaceutical industry, as well as contribute to the development of personalized medicine. It is currently well accepted that consideration of pharmacogenetics is essential in the production and administration of therapeutics. With our growing knowledge of the functions and composition of the microbiota, the concept of pharmacogenetics should be expanded into pharmacometabonomics, which would include the contribution of both the host and the microbiota to the metabolism of pharmaceuticals. An interesting "Pachinko model" has been put forward which explains drug interactions and therapy outcome in terms of probabilistic interactions of the administered compound with the host and microbial metabolic pathways and the concurrent presence/absence of additional compounds competing for the same metabolic pathways (213). In this model, most probable interactions lead to generation of metabolites that promote the expected therapy outcome, while less probable interactions (due to unusual host genome polymorphisms, inherently unusual or perturbed microbiota composition or the presence of interfering compounds) lead to the generation of metabolites that promote idiosyncratic reactions to therapy.

Dietary modifications are often suggested for preventative and therapeutic purposes in a wide range of conditions. For instance, it has been observed that phenolic compounds have the ability to reduce or reverse the development of colitogenic changes at the intestinal mucosa, thus offering a prophylactic and sometimes a therapeutic means against colorectal carcinomas (3). However, when evaluating the efficacy of a given dietary intervention, such as a diet rich in phenolics, the efficacy of microbiota-produced metabolites of the parent compound needs to be evaluated along with the efficacy of the parent compound itself. Russell et al. (252) have demonstrated that the anti-inflammatory potential of phenolic metabolites is often reduced compared with the parent compounds. Consequently, the individual's microbiota composition and its ability to biotransform nutritional compounds with potential medicinal significance should be considered when recommending dietary interventions.

In addition to the microbiota's contribution to metabolism of medicines administered with therapeutic purposes, it also has the ability to metabolize certain dietary compounds into metabolically active forms that proceed to influence various aspects of host health. For instance, gut *Bifidobacterium* strains conjugate dietary linoleic acid (223), which has a wide variety of biological effects (45). Oral microbiota was required for reduction of dietary nitrate to biologically active nitrite (235). Additionally, *Oxalobacter formigenes* has the ability to degrade dietary oxalates, reducing urinary oxalate excretion (275), which prompted its successful use in clinical trials as a therapeutic and prophylactic option in calcium oxalate nephrolithiasis and associated renal failure (124). Furthermore, gut inhabitants can prove invaluable in preventing adverse outcomes following inadvertent environmental exposure to toxic compounds: the toxicity of hydrazine, a highly toxic compound used in a variety of industrial processes, is greatly reduced by the gut microbiota (302).

F. Concluding Remarks

As our knowledge and understanding of gut microbiota function in postnatal development of the host continues to expand and improve, it provides the opportunity to ask more refined questions about host-microbiota interactions and design new and exciting hypothesis-driven studies. The realization that even just the presence of bacterial structural components or metabolites can be sufficient to induce development and maturation of different host organs and processes should also prompt increased rigor in quality control of experiments involving GF animals. It has been shown, for instance, that contamination of sterile diet with bacterial LPS is sufficient to trigger some aspects of immunological development in GF mice, both at the intestinal mucosa and systemically (125), an event that has the potential to confound and skew data interpretation obtained from comparison of GF and mono- or poly-associated animals.

IV. MICROBIOTA IN DISEASE: MECHANISMS OF FINE BALANCE

Dysregulation of the intestinal mucosa homeostasis leads to a multitude of ailments, from the obvious case of inflammatory bowel diseases (IBD) (117, 262) to the more unexpected activation of chronic human immunodeficiency virus (HIV) infection (115) and generation of atopy (168, 232, 322). As the intestinal microbiota is a key purveyor of the mucosal homeostasis, it is consequentially implicated in the progression of these disorders.

A. Imbalance Leads to Chaos

The importance of microbial balance is readily appreciated when considering some of the deleterious sequelae of antibiotic treatment. Several studies have shown the adverse effects of different antibiotics on the host gut microbiota in both human subjects (54, 58, 135, 171, 304) and animal models (8, 51, 272, 344). The aftermath of antibiotic administration often lasts for a long time after discontinuation of treatment (51, 58, 135, 167, 171), suggesting that a prolonged dysfunction is induced in the host's microbial "organ."

One of the best known complications arising following antibiotic therapy is antibiotic-associated diarrhea, which can be due to the pathological overgrowth of *Clostridium difficile* in the antibiotic-treated GIT (199). The extent of the perturbation in the gut microbiota community of human patients was shown to be linked to the likelihood of developing recurrent *C. difficile* infection (41), while antibiotic treatment of mice carrying *C. difficile* increases shedding of *C. difficile* spores and promotes transmission to uninfected hosts (157). Treatment of *C. difficile*-associated diarrhea with a combination of vancomycin and a yeast probiotic was shown to be more effective than vancomycin alone in preventing recurrence (236, 299). Additionally, administration of a bacterial probiotic to healthy volunteers taking amoxicillin resulted in a significant reduction of incidence of diarrhea in study subjects (149), demonstrating that attempts to rebalance the gut microbiota diminish occurrence of antibiotic-associated diarrhea. Vancomycin-resistant *Enterococci* (VRE) are another example of an opportunistic pathogen of particular concern in the hospital setting (170). A recent animal study has shown that they are able to exploit an immune deficit (downregulation of RegIIIγ, an antimicrobial peptide with Gm+ spectrum of activity) created at the intestinal mucosa by antibiotic-mediated disruption of the gut microbiota to prolong their colonization of the infected murine host (28). In addition to predisposing the host to colonization by opportunistic *C. difficile* and VRE, antibiotic-induced microbiota disturbances were also shown to predispose the host to a higher risk of nontyphoidal *Salmonella* infection (97), demonstrating that both opportunists and pathogens alike are able to benefit from dysbiosis in the GIT.

The use of antibiotics in neonates has a positive correlation with increased risk of intestinal intussusception (130), demonstrating the increased danger of pediatric microbiota disturbances. Disturbances in microbiota composition can also adversely affect function of multiple host organs, as increased reduction of gut microbial diversity concurrent with overgrowth of *Enterococci* in critically ill patients following treatment in an intensive care unit was shown to correlate with greater organ failure and mortality (131).

All these studies point to the dangers inherent to the overuse of antibiotics in the clinical setting. Disturbance in the gut microbial communities created by the administration of antibiotics has the potential to adversely affect the function of multiple host organ systems for a prolonged period of time, as it takes time for the microbial community to return to status quo. Additionally, exposure of the microbial inhabitants of the GIT to various antibiotics is likely to result in development of various resistance patterns, which can further delay or prevent the return to equilibrium following repeated administration of the same antibiotic regimens. Furthermore, it can promote the spread of antibiotic resistance among pathogenic bacteria that will come in contact with the antibiotic-resistant microbiota.

B. Microbial Intruders of the GIT

Despite all the protective mechanisms present at the gastrointestinal mucosa, humans occasionally fall victim to invading enteric pathogens, including bacteria and viruses. To establish a successful infection at the GIT, enteric pathogens need to be able to penetrate through a dense bacterial population overlying the intestinal mucosa. Their means to this end are sometimes surprising and almost counterintuitive (231), and the invariable outcome of the infection is a disturbance in the host's gut microbial community, which has the capacity to predispose the host to further unpleasant postinfectious sequelae (271) (Fig. 4).

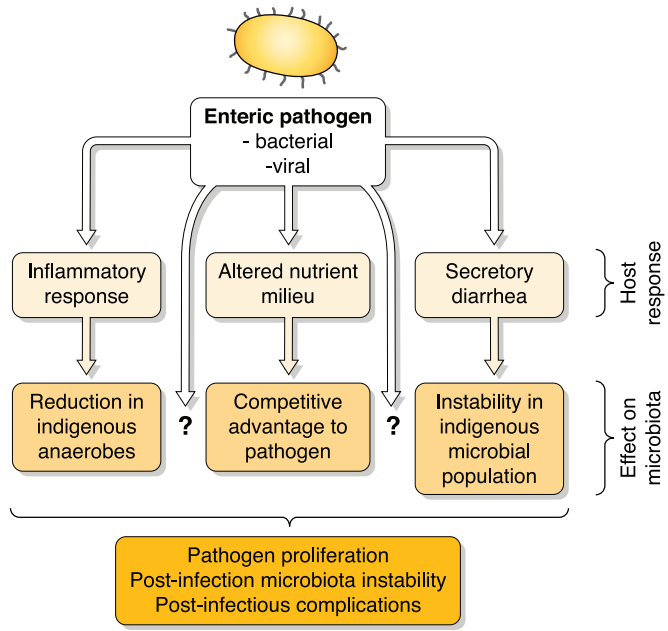


FIG. 4. Pathogen-host-microbiota interactions and outcome of infection. Enteric pathogens utilize their arsenal of virulence factors to evoke a host response that destabilizes the indigenous microbiota and adversely affects both its protective and immunomodulatory functions. Additionally, the pathogens are likely to interact directly with the microbiota through, as of yet, unknown mechanisms (arrows leading to question mark). As a result, the pathogen is able to proliferate, further impacting on both the host and the microbiota, resulting in postinfection microbiota instability and postinfectious complications.

1. *Inflammation-mediated effects on the microbiota*

Colonization of the intestinal mucosa by bacterial enteric pathogens results in the induction of a strong inflammatory response aimed at controlling the offending pathogen. However, this inflammatory response has also been shown to have the unexpected effect of decreasing the viability of the gut microbiota, allowing the pathogen to occupy the vacated niches (231, 271, 287, 290).

A number of pathogens, all from the Proteobacteria phylum, have been shown to utilize the approach of inflammation-mediated assault on the microbiota to maximize their infective potential. *Citrobacter rodentium*, a murine equivalent of pathogenic *E. coli*, and *Salmonella enterica* serovar Typhimurium, both of the *Enterobacteriaceae* family of the γ-Proteobacteria class, cause an inflammation-mediated reduction in total numbers of gut microbiota, facilitating their successful establishment in the GIT (175, 291). *Helicobacter trognotum*, a murine pathogen in the ϵ-Proteobacteria class, was shown to cause more severe GIT pathology when infecting IL10−/− mice predisposed to inflammation (331). The composition of the host's microbiota was altered by the infectious process (15, 116, 175, 272, 291); in particular, the resident obligately anaerobic bacteria were adversely affected (15, 175, 272, 291, 331). At the same time, inflammation at the intestinal mucosa was shown to promote the overgrowth of *Enterobacteriaceae*, commensal as well as pathogenic (175). Interestingly, probiotic-mediated reduction of local and systemic inflammatory host response was shown to provide the host with some protection during *S.* Typhimurium infection (222), further demonstrating the importance of inflammation at the intestinal mucosa to pathogen fitness.

Several theories regarding the factors contributing to the enhanced pathogen fitness in the inflamed GIT have been suggested (290). A recent study by Stecher et al. (289) provided support to one such theory, which proposes that the invading pathogen is better able to utilize the nutrients available in the inflamed intestine than the gut microbiota (289). *S.* Typhimurium was shown to utilize its motility to benefit from mucins released during inflammation, enhancing its growth and consequently fitness. Additionally, it was shown that treatment of mice with a high dose of streptomycin, which facilitates the induction of *S.* Typhimurium-mediated intestinal pathology, greatly alters the fatty acid composition of mouse ceca (83) where *S.* Typhimurium infection and pathology center, further suggesting that the gut nutritional milieu can either promote or inhibit infection.

2. *Viral enteric infections and postinfection complications*

Viral infections of the GIT, specifically rotavirus infections, are the most common cause of pediatric diarrhea worldwide (52). Rotavirus infections also have the capacity to adversely affect the host's gut microbiota: alterations in the composition of the *Bacteroides* community were observed in rotavirus-mediated diarrhea (348). The observed alterations were likely a collateral damage resulting from the diarrhea-associated disturbance of host intestinal homeostasis, rather than a result of direct interactions between the rotavirus and the resident bacteria, as another study found similar gut microbiota alterations in both rotavirus and nonrotavirus diarrhea (13). Probiotic treatment was shown to be particularly effective for rotavirus diarrhea (274), demonstrating that a healthy gut microbial community provides the host with protection against rotavirus infections. It would be very interesting to determine whether viral enteric pathogens strive to maximize their fitness in the GIT by orchestrating host-mediated assault on its gut microbiota, or the observed microbial disruption is just the collateral damage of the virus's attempts to maximize its dispersion.

Irritable bowel syndrome (IBS), a disorder of the GIT with complex etiology, sometimes develops following recovery from enteric infections (286), suggesting that infection-mediated disturbance in the host's gut microbial community results in GIT malfunction. Postinfectious IBS in animal models can be ameliorated with the administration of probiotic bacteria, which normalize muscle hypercontractility (176), offering further evidence that postinfection microbiota disturbance contributes to IBS pathophysiology.

C. Disorders of the GIT

1. *IBD*

IBD, which includes Crohn's disease (CD) and ulcerative colitis (UC), has long been suspected to involve an aberrant host response to its gut microbiota. This theory was supported by a certain degree of effectiveness of antibiotics in the prevention and treatment of colonic inflammation in both human patients and animal models, as well as by the presence of microbes and microbial components in inflammation-induced colonic lesions (262). Many aspects of the microbiota's involvement in IBD have been expertly reviewed over the last few years (226, 233, 261, 262, 287, 340).

While aberrant gut microbiota has been noted in both human IBD patients (75, 76, 233, 262) and in animal models of intestinal inflammation (175), the cause and consequence relationship of abnormal microbiota and IBD development has always been the subject of debate. More specifically, several questions arise regarding the involvement of gut microbiota in IBD pathogenesis. Is the immune-mediated damage due to recognition of particular bacterial epitopes or to molecular mimicry-mediated autoimmune reaction? Is the aberrant immune response due to the presence of a particular immunogenic species of microbiota, or to a microbiota imbalance in which more colitogenic members are present? Would the transfer of colitogenic microbiota produce colitis in previously healthy hosts? Can certain microbiota members or bacterial components promote a tolerogenic host response? Would it only be helpful as a preventative strategy, or can it also ameliorate already established colitis? Recently, mechanisms underlying some aspects of microbiota-mediated injury to the GIT have been elucidated (Fig. 5), enhancing our understanding of the IBD pathogenesis and laying ground for the design of more specific and effective therapeutics.

While some studies have suggested a role for autoimmune reactions resulting from bacterial-host mimicry in the pathogenesis of IBD, the majority of potential mimickers belong to pathogenic bacteria, such as *Mycobacterium spp.*, *Campylobacter*, and *Klebsiella* (262). While commensal bacteria can be recognized by perinuclear antineutrophil cytoplasmic autoantibodies (pANCA), this recognition does not appear to contribute to tissue damage in IBD (262). The idea that an immune reaction to a particular bacterial epitope can precipitate colitogenic changes at the intestinal mucosa was elegantly confirmed by Kullberg et al. (153), who have shown that transfer of a single CD4+ Th1 T cell clone specific to a particular bacterial epitope to RAG−/− mice (lacking T and B lymphocytes) infected with the bacterium recognized by these T cells induced colitis in the recipient mice. The inflammatory response developed specifically in the presence of a bacterium that could be recognized by the transferred T cells, as uninfected mice or mice infected with an unrelated pathogen remained colitis free. The authors of the study used *Helicobacter hepaticus* as the immunogenic bacterium (153), which is a murine pathogen rather than a component of the normal murine gut microbiota. Therefore, a question remained as to whether or not recognition of an indigenous bacterium would be able to instigate colitis. It was shown that reconstitution of SCID mice (also lacking T and B lymphocytes) with $CD4+CD45RB^{high}$ T cells from conventional (CV) mice produces colitis in the recipient host, but only in the presence of particular components of the normal gut microbiota (60, 293). While colitis developed in mice mono-associated with bacterial species that have pathogenic potential, mono-association with SFB bacteria, a member of the gut microbiota, did not result in inflammatory changes upon T-cell transfer (60, 293). Conversely, association of mice with a defined bacterial cocktail consisting of a number of other indigenous bacteria in addi-

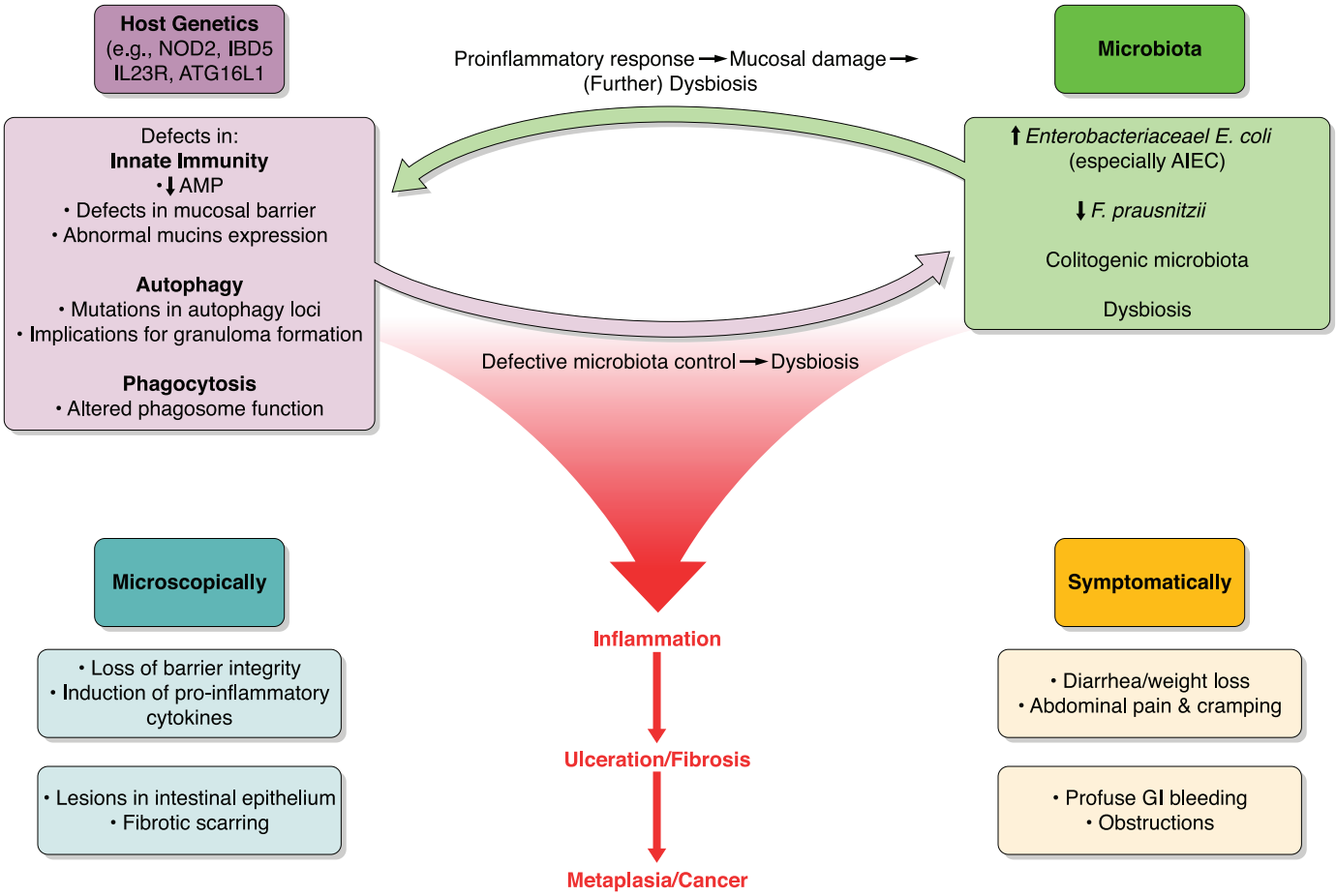


FIG. 5. The role of microbiota in inflammatory bowel disease (IBD) pathogenesis. The interplay between the host microbiota and host genetics (as well as environmental stimuli not included in the figure) results in progressive inflammatory damage to the host's intestinal mucosa, manifested through characteristic histopathological findings and associated symptomatology. Top purple boxes include examples of host genetic deficits associated with IBD. Top green boxes include examples of anomalous findings from gut microbial communities of IBD patients and animal models. Bottom blue boxes give examples of histopathological findings in IBD. Bottom light yellow boxes give examples of symptoms manifested by IBD patients. Red arrows highlight a possible clinical course of IBD progression.

tion to SFB proved to be colitogenic in the presence of transferred T cells (293). Thus it appears that concurrent recognition of multiple members of the gut microbiota is necessary to initiate inflammatory changes at the intestinal mucosa. The inflammation was suggested to be associated with detrimental changes at the intestinal epithelium, such as loss of barrier integrity (293) and induction of inducible nitric oxide synthase (60).

Although no single member of the gut microbiota responsible for the instigation of IBD in otherwise nonpredisposed hosts has been identified to date, several studies have shown a high incidence of pathogenic *E. coli* in ileal biopsies of CD patients (226, 262, 287). This adherent/invasive *E. coli* (AIEC) strain binds to CEACAM6, which is expressed on the apical surface of ileal epithelial cells, and is upregulated in CD (262). Intracellular invasion by AIEC is associated with active CD and intestinal pathology. Interestingly, AIEC associated with CD was shown to be closely related to uropathogenic, rather than enteropathogenic *E. coli*, possibly indicating that it represents a phenotypically altered (probably through horizontal gene transfer) member of the gut microbiota, rather than an infecting pathogen. *Mycobacterium avium* subspecies *paratuberculosis* is another bacterial species that has been often linked to CD etiology, but with no conclusive evidence to its involvement being produced to date (226). While single culprits responsible for IBD have been hard to find, numerous studies have shown an alteration to the composition of the gut microbial community in IBD, both CD and UC (233). The majority of these studies have reported a relative abundance in *Enterobacteriaceae* in IBD patients, and recently, a decrease in the numbers of *Faecalibacterium prausnitzii* was shown to be associated with and highly indicative of CD localized to the ileum (278, 336). However, as mentioned previously, it is still unclear whether the IBD-associated alterations in the gut microbiota are the cause or the consequence of the disease. One recent study indicates that "colitogenic" microbiota may be both: it can result from collateral damage of mucosal inflammation and also promote fur-

ther pathology (84). Garrett et al. (84) have shown that RAG2−/− mice that are also deficient in T-bet, a transcription factor responsible for regulating inflammatory responses, are susceptible to spontaneous UC that can be cured by administration of broad-spectrum antibiotics that suppress anaerobic microbiota, indicating that these mice appear to harbor abnormal colitogenic microbiota. Thus a genetic predisposition to inflammation was suggested to affect microbiota composition. Even more interestingly, when this colitogenic microbiota was transferred to T-bet sufficient RAG2−/− or WT mice, these mice also developed colitis, demonstrating that abnormal microbiota was able to initiate an inflammatory response in individuals not genetically predisposed and highlighting the fact that colitis can be communicable (84).

It is of interest to note that while in certain circumstances gut microbiota contributes to IBD pathogenesis, overt inflammation at the intestinal mucosa appears not to be beneficial to it as a whole. It has been shown that vitamin D, which is produced by the microbial inhabitants of the GIT, acts to decrease the severity of dextran sodium sulfate (DSS)-induced intestinal inflammation in an animal model of colitis (79). In addition to underscoring the ultimate importance of mucosal homeostasis, this finding also points to another potential therapeutic option for IBD.

Numerous attempts have been made to treat and/or prevent IBD through the use of pre- and/or probiotics, aiming to redress the inflammation-promoting imbalance in gut microbiota. Some studies report success of pre- and/or probiotics administration in ameliorating colitis symptoms or preventing postoperative recurrence, while others have seen fewer benefits (226, 262, 340). To further our knowledge and effectiveness of pre-/probiotic treatments of IBD, future studies need to focus on mechanisms underlying their mode of action. Interestingly, administration of *F. prausnitzii* that, as described above, appears to be lacking in several IBD states, was shown to reduce the severity of trinitrobenzesulfonic acid (TNBS) colitis (278). *F. prausnitzii* was shown to minimize secretion of proinflammatory cytokines and to increase secretion of IL-10 both in vivo and in vitro, as well as to ameliorate the dysbiosis observed in colitic mice (278), offering insight into some potential mechanisms underlying its therapeutic effect.

It should also be stated that while the gut microbiota is undoubtedly a central participant in IBD pathogenesis, so is the host genotype. Numerous studies have implicated multiple genetic loci in IBD pathophysiology (reviewed in Refs. 262, 340). Hosts carrying many of these loci appear to be more prone to GIT inflammation due to the lack of proper regulation of their gut bacterial communities, as well as due to an overzealous inflammatory response to their resident microbes.

2. *GIT malignancies*

While the gut microbiota is an essential partner in health, some aspects of its presence can induce highly unwanted carcinogenic processes in the host. The proposed mechanisms of microbiota-induced carcinogenesis generally fall into three categories. Disproportionate proinflammatory signaling at the GIT mucosa (such as that induced by the presence of an excessively colitogenic microbiota) results in increased sloughing and repair of the intestinal epithelium. This process can ultimately result in the formation of neoplasia and malignancy. Alternatively, certain microbial species can have direct cytotoxic effects on cells at the intestinal mucosa or cause toxicity through a bystander effect (in which host tissue can be damaged by host cells activated by certain microbial species). Additionally, metabolism of some nutrients by particular members of the microbiota can elaborate by-products that are toxic to the intestinal epithelium. Imperfect repair of the injured epithelium can then result in neoplastic transformations.

Perhaps the best-known and most-studied example of a microbiota-induced GIT malignancy is the *Helicobacter pylori*-mediated gastric carcinoma (49) (Fig. 6*A*). About half of the world's population carries *H. pylori* (49), so this bacterium can be thought of as part of the gastric microbiota. Its presence induces a persistent immune response in the host, resulting in a state of inflammation at the gastric mucosa that ultimately leads to malignant transformations at the gastric epithelium. *H. pylori*-induced carcinogenesis has been proposed to proceed through a number of avenues. Murine models of *H. pylori* infection, utilizing *H. felis*, have shown that induction of a T cell-mediated response (251) and a Th1 cytokine milieu (205) are necessary for the bacterium-mediated pathology to develop, demonstrating the importance of host-microbiota bidirectional interactions in producing either the outcome of health or disease. Additionally, *Helicobacter* was shown to induce production of reactive nitrogen intermediates at the gastric mucosa (188), which has the potential to produce carcinogenic DNA damage (16). Regulators of DNA transcription were also shown to be affected by *H. pylori* (191).

Interestingly, *H. pylori* isolates from the same human host, one during the stage of chronic atrophic gastritis (which precedes gastric neoplasia) and the second following malignant transformation at the gastric mucosa, were shown to interact in distinct ways with gastric epithelial stem cells (87). Thus evolution towards tumorigenesis at the gastric mucosa involves adaptations on both the host's and the bacterium's side. Eradication of *H. pylori* through antibiotic therapy and suppression of gastric acid secretions resolves the gastric inflammation, consequently making *H. pylori*-associated carcinomas one of the most preventable cancers. However, the cost of

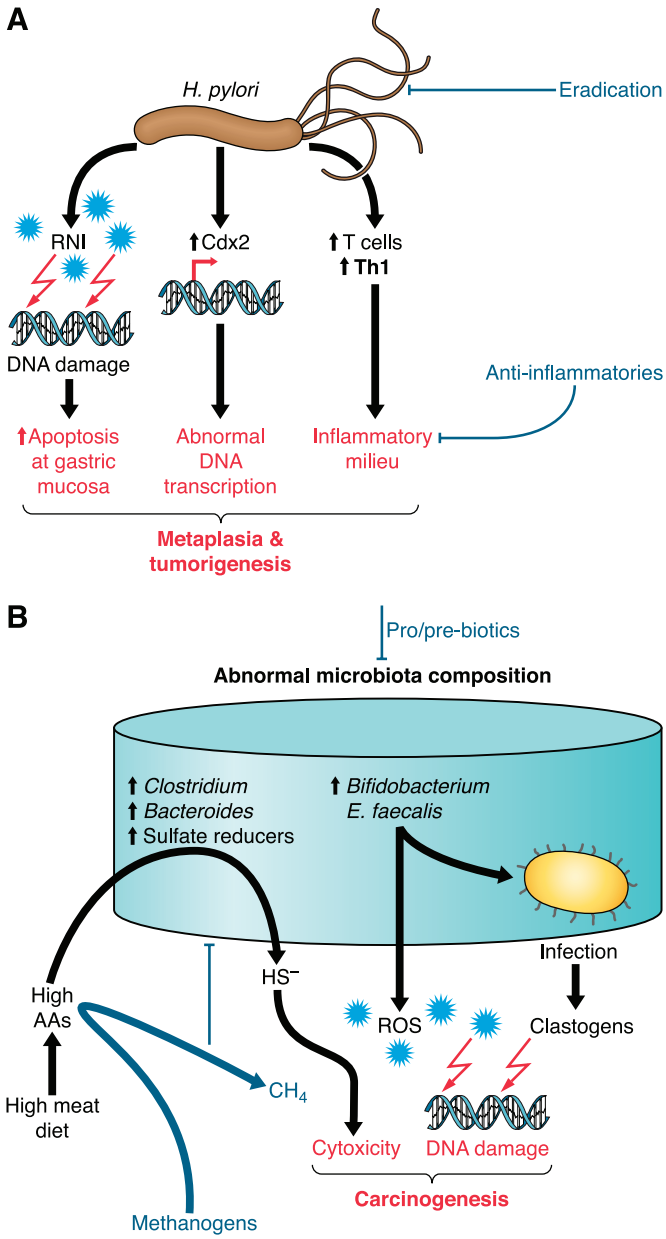


FIG. 6. Microbiota-dependent carcinogenesis. *A*: *H. pylori*, a causative agent of gastric cancer, induces carcinogenic processes through direct damage to host DNA [caused by production of reactive nitrogen intermediates (RNI)], dysregulation of DNA transcription factors (e.g., Cdx2), and induction of inflammatory milieu at the gastric mucosa. Blue blunt arrows indicate possible therapeutic intervention points, such as eradication of *H. pylori* to prevent future metaplastic transformations and the use of anti-inflammatory compounds to correct ongoing metaplastic processes. *B*: intestinal carcinogenesis can be a result of dysbiosis in the colonic microbiota, with an increased proportion of bacteria whose metabolism elaborates cytotoxic compounds (e.g., sulfate reducing bacteria) or bacteria which cause DNA damage either through production of free radicals [e.g., reactive oxygen intermediates (ROS)] or through abnormal activation of resident immune cells (e.g., macrophages). Blue blunt arrows indicate possible therapeutic intervention points, such as correction of overall dysbiosis (pre/pre-biotics) or selective seeding of bacterial populations whose metabolism is not damaging to the host (e.g., methanogenic bacteria).

worldwide *H. pylori* eradication may be substantial from both the economic and the biological points of view; loss of a commensal that has a long history of codiversification with its human host may potentially be accompanied by a loss of some of its currently unknown benefitial effects. Studies comparing digestive function of people naturally colonized by *H. pylori* to those lacking this commensal might be able to shed some light on this point.

Different microbiota members have also been implicated in the development of colorectal carcinomas (123) (Fig. 6*B*). Gut microbiota composition is altered in colon carcinoma patients (206, 265). Carcinoma-associated microbiota was characterized by an increase in the diversity of *Clostridium spp.* (265), as well as enriched for *Bacteroides and Bifidobacterium spp.* (206). Conversely, the microbiota in a cohort of people at low risk for colorectal carcinoma development was shown to be abundant in lactic acid-producing bacteria, such as *Lactobacillus spp.* and *Eubacterium aerofaciens* (206). Interestingly, the microbiota composition of polyposis (which generally precedes the development of a carcinoma) patients was also different from that of controls, but similar to that observed in colon carcinoma (265). This demonstrates that alterations in gut microbiota likely precede the onset of malignant transformations, although it remains to be determined whether the abnormality in microbiota drives carcinogenesis or host- and/or diet-mediated carcinogenic alterations in the colonic environment promote concurrent alterations to the resident microbial community.

Epidemiological evidence suggests that diet is a major influence in the development of colorectal malignancies (21), and consequently, it was proposed that microbial metabolic by-products of dietary compounds could produce either a cytoprotective or a cytotoxic effect at the intestinal mucosa (123, 221). One of the mainstream hypotheses proposed that the concentrations and composition of microbiota-produced SCFAs would be altered in the carcinogenic colons, with consequent adverse effects on the maintenance of the colonic epithelium (221). However, a recent study showed no difference in colonic SCFAs between colonic carcinoma patients and control subjects (265), demonstrating that SCFAs are unlikely contributors. Another theory postulates that increased production of toxic compounds, such as hydrogen sulfide, by the microbiota could produce cytotoxic and consequently carcinogenic effect (123, 221). Hydrogen sulfide can be produced through metabolism of amino acids, which are the products of digestion of dietary proteins, and a high-protein diet has been linked to increased incidence of colon cancer (221). In fact, an increased abundance of amino acids was demonstrated in fecal water of colon carcinoma patients (265). Populations at low and high risk for colorectal cancer have been shown to harbor methanogenic and nonmethanogenic microbiota, respectively (221). Methanogenic microbiota preferentially pro-

duces harmless methane as an end-product of amino acid metabolism, while nonmethanogenic microbiota that is enriched in sulfate-reducing bacteria would result in excessive elaboration of highly toxic hydrogen sulfide (88). Further studies are required to conclusively demonstrate the link between microbiota with a high proportion of sulfate-reducing bacteria, a high-protein diet, and carcinogenesis in the GIT. However, if causation was established, modification of microbiota towards a methanogenic state could be a useful preventative strategy in populations at high risk for colon carcinoma.

In addition to the production of toxic by-products of dietary nutrients, the metabolism of some members of the colonic microbiota results in the elaboration of toxic elements, irrespective of nutrient source. For instance, *Enterococcus faecalis* produces extracellular reactive oxygen species (ROS) that have been shown to induce DNA damage in colonic epithelial cells, both in vitro and in vivo (129). Direct exposure of colonic epithelial cells to *E. faecalis* or *E. faecalis*-produced ROS was not necessary for damage to occur, as it was also observed in colonic epithelial cells cocultured with macrophages harboring *E. faecalis* (327). Macrophages activated by *E. faecalis* were shown to produce DNA-damaging clastogens, exposure to which resulted in DNA damage of colonocytes, a phenomenon called the bystander effect (327).

Inflammation-promoted tumorigenesis also plays a significant role in colorectal tumor development, and it has been noted that patients suffering from IBD are at an increased risk of colon cancer (189). Just as the gut microbiota plays a major role in the development of IBD, it has also been shown to contribute to colitis-associated colorectal carcinoma development. IL-10−/− mice, which develop spontaneous colitis when colonized with normal gut microbiota, had a very high incidence of colorectal carcinomas after exposure to a potent carcinogen (316). Conversely, exposure to a carcinogen of GF IL-10−/− mice did not result in malignant colonic neoplasias, whereas IL-10−/− mice mono-associated with a mildly colitogenic bacterium had a reduced incidence of colorectal carcinoma development following exposure to a carcinogen, compared with mice colonized by normal gut microbiota (316).

It can be challenging to study the role of the host microbiota in colorectal carcinogenesis, as in the human host it is a life-long process, occurring over many decades, a situation that cannot be simulated by the rodent animal models. However, as our knowledge of the microbiota component in this pathological process continues to expand, new promising avenues of therapeutic and prophylactic interventions (such as dietary and microbiota modifications) will emerge, hopefully helping us gain an upper hand in the battle with this unduly common and fatal malignancy.

D. Disorders of the GIT Accessory Organs

1. Cholelithiasis

An involvement of the gut microbiota in the formation of gallstones has recently been demonstrated. Abnormal metabolism and secretion of cholesterol and bile acids are a primary pathophysiological defect in the formation of gallstones, with intestinal hypomotility and chronic inflammatory changes being contributing factors (326). As discussed previously, gut microbiota plays a central role in the regulation of all of these processes and, as such, is indirectly implicated in cholelithiasis. To demonstrate this, contamination of lithogenic bile with members of the gut microbiota has recently been shown (1, 37). The majority of the cultured bacteria belonged to *Enterobacteriaceae*, and some isolates to *Enteroccocus* and *Streptococcus* genera (1, 37). The presence of gut microbiota members in lithogenic bile could be an indication of increased intestinal permeability during biliary obstruction (332), likely contributing to increased inflammatory response and stone formation. Alternatively, bacterial contamination could instigate lithogenesis by inducing cholestasis.

2. Liver disease and minimal hepatic encephalopathy

Endotoxemia causes inflammation that is responsible for the symptoms associated with liver diseases, such as cirrhosis of the liver. Portal hypertension, the instigator of liver disease, can also result in increased intestinal permeability and consequent endotoxemia. The resulting systemic inflammation can prove deleterious to various organs, including the liver (216). Studies in cirrhotic rats have shown that therapeutic administration of insulin-like growth factor (IGF-I), a growth factor responsible for gut barrier maintenance, is able to limit the development of liver cirrhosis in animals with liver disease by enhancing intestinal barrier function and reducing levels of bacterial translocation (173). Fecal flora analysis of cirrhosis patients revealed reduced levels of beneficial *Bifidobacteria* (349). Minimal hepatic encephalopathy (MHE) is a complication of cirrhosis during which accumulation of neurotoxic substances in the bloodstream produces neurological manifestations. As cirrhosis has been linked to changes in the gut microbiota, one group investigated the effects of probiotics and/or prebiotics on MHE. MHE patients were given a synbiotic, a prebiotic, or a placebo. Interestingly, MHE was reversed in 50% of the patients that received the synbiotic and in some who received the prebiotic alone. This effect was accompanied by a significant increase in nonurease producing *Lactobacilli* (169).

E. Complex Multifactorial Disorders and Diseases of Remote Organ Systems

Recent research efforts focused on elucidating the contributions of the gut microbiota to the etiology of human diseases have begun to shed light on its involvement in a considerable number of complex diseases residing in organs outside (and far from) the gut (Fig. 7).

1. *Obesity*

Considering the previously presented discussion on the importance of microbiota for nutrient acquisition and production of vitamins and other bioactive molecules, it should not be surprising that research into the role of microbiota in the development of obesity has yielded interesting results.

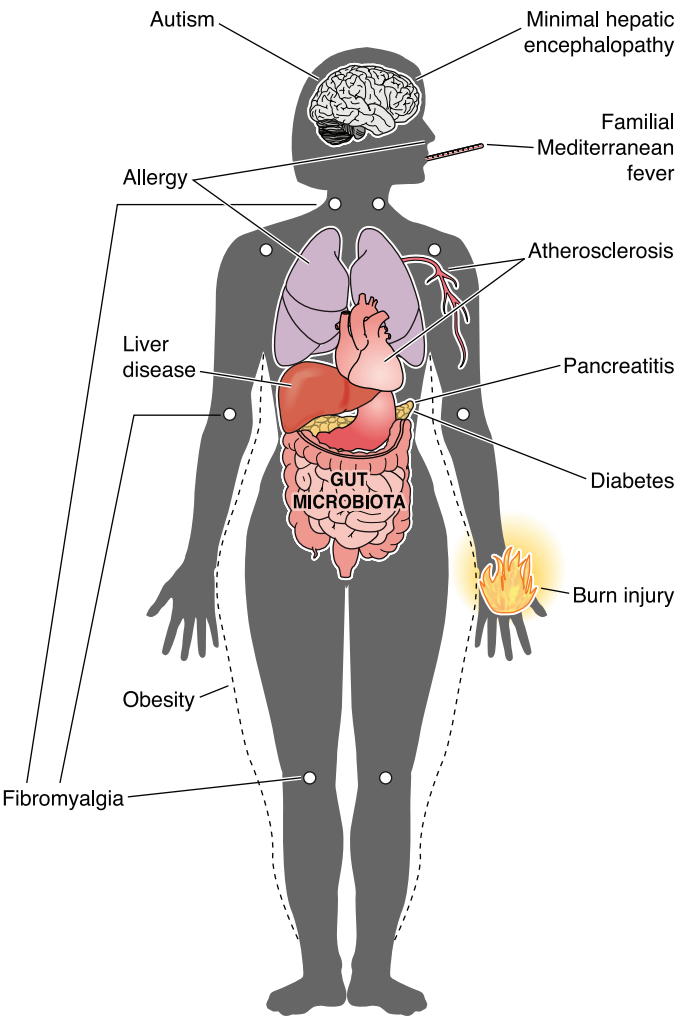


FIG. 7. Association of microbiota with diseases outside of the gastrointestinal tract. The intestinal microbiota has been linked to a number of diseases that are associated with remote organ systems. Solid arrows indicate the organ systems affected by the diseases indicated. Obesity is indicated by a dashed black line, and fibromyalgia is illustrated by the characteristic pressure points typically used to diagnose the disease (white dots).

In recent studies, the group of J. I. Gordon was able to demonstrate that the gastrointestinal microbiota is directly involved in the regulation of energy homeostasis in murine and human hosts. Analysis of more than 5,000 16S rRNA sequences revealed that genetically obese mice (*ob/ob*, characterized by a mutation in the leptin gene) had a 50% reduction in the abundance of Bacteroidetes, and a correspondent increase in the proportion of Firmicutes, compared with lean (*ob/+* and +/+) mice. Comparisons of obese and lean distal gut microbiotas from mice and humans indicate that the community structures are very similar at the division level (161). Microbiota profiling in humans indicates that the Bacteroidetes-to-Firmicutes ratio in obese individuals put on a "lean" diet increases towards that which is expected in a "lean" individual (163). As over 90% of the phylogenetic types ("phylotypes") present in the gut microbiota belong to the Firmicutes and the Bacteroidetes divisions, the dramatic shift in microbiota composition seen in obese individuals indicates that substantial changes are being made to the functional gut ecosystem. Indeed, characterization of the gut microbiome of obese mice (*ob/ob*) and their lean (*ob/+* and +/+) littermates showed that compared with the "lean" microbiome, the obesity-associated microbiome harbors a substantial increase in genes encoding enzymes involved in the breakdown of dietary polysaccharides (313). In agreement with this, a significantly lower amount of energy remained in the feces of obese mice, relative to their lean counterparts, highlighting the increased capacity of the obese microbiome for energy extraction from the diet (313). The increase in polysaccharide-degrading enzymes found in the obese microbiome was also linked to increased fat deposition using a microbiota transplantation model. GF mice were transplanted (i.e., conventionalized) with microbiotas from either obese or lean donor mice, and it was shown that mice conventionalized with "obese microbiota" had significant increases in body fat relative to mice conventionalized with "lean microbiota" (313). Additionally, GF mice conventionalized with microbiota from donors with diet-induced obesity (DIO) (i.e., mice that were fed a "Westernized" diet) also had increased body fat deposition. However, this DIO microbiota phenotype was reversible upon discontinuation of the "Westernized" diet. Interestingly, mice that were fed a Westernized diet but remained GF were protected from the obese phenotype (311).

2. *Allergy*

Decades have passed since Strachan first proposed the "hygiene hypothesis," a theory that suggests that factors influencing the extent of an individual's exposure to pathogenic microorganisms such as household size, sanitation, birth order, and antibiotic use are the reasons why the prevalence of atopic diseases like eczema, hay fever,

and asthma have drastically increased in countries that have adopted the "Westernized" way of life (295). Recently, several groups have suggested that this hypothesis should be revised to incorporate a role for the gastrointestinal microbiota. The "microflora hypothesis," initially put forth by Noverr and Huffnagle (218), suggests that perturbations in the gastrointestinal microbiota as a result of reduced microbial exposure due to changes in diet and antibiotic use result in an underdeveloped microbiota. This "immature" microbiota delays proper maturation of the immune system, disrupting the normal sequence of events that promote the development of immunological tolerance, increasing the incidence of allergic hypersensitivity (218). There have been a number of epidemiological studies conducted that investigate correlations between atopy and tenants of the hygiene hypothesis (e.g., birth order, household size, antibiotic usage, etc.) (156, 294). Several birth cohort studies have indicated associations between antibiotic use and increased risk of allergy (200) and asthma (322, 337), emphasizing that allergic symptoms positively correlate with earlier antibiotic administration and increased dosage. Other groups tried to directly associate particular trends in the composition of the intestinal microbiota to demonstrable allergic symptoms. Several prospective studies have used culture-based methods to conclude that significant reductions in certain groups of the microbiota are correlated with allergic symptoms early in life. The levels of *Bifidobacterium* and *Enterococcus spp.* were shown to correlate with allergic symptoms in the first month of life. However, by 6 mo of age, *Enterococcus spp.* levels returned to those observed in healthy infants (23). A higher Bacteroidetes-to-*Bifidobacterium spp.* ratio was reported at 2 yr of age in infants that showed symptoms of atopy (301). Culture-based methods of microbiota analysis definitely have their limitations, possibly accounting for common inconsistencies. Quantitative real-time PCR (qPCR) revealed that children who developed allergies were less frequently colonized with *Lactobacillus spp.*, *Bifidobacterium spp.*, and *Clostridium difficile* at 2 mo of age and that children belonging to larger households consistently generated more diverse microbiota profiles (276). Similarly, *Clostridium spp.* appears to be associated with protection against wheezing during the first year of life (322).

In addition to epidemiological studies, from which it is often difficult to obtain consistently conclusive data, animal models of allergy have been generated to investigate the biological aspects of allergy development. To explore the development of cow's milk allergy (CMA), one of the most common allergies among infants (32), β-lactoglobulin (BLG) sensitization was investigated in GF, SPF, and SOPF (specific and opportunistic pathogen free) BALB/c mice. Despite the common antigen, the immune response differed in the three different types of mice. Serum BLG-specific IgG1 and IgE concentrations were significantly higher in GF mice during the primary response, and IgE persisted for longer (106). Alternatively, Huffnagle and colleagues (217, 219) have developed a fungal spore model to gain insights into respiratory-related allergy. Antibiotic treatment was combined with oral administration of *Candida albicans* to promote enhanced fungal colonization in the gut. When these antibiotic-treated mice were exposed to fungal spores (*Aspergillus fumigatus*), they demonstrated more severe allergic airway disease than their untreated counterparts, evidenced by significant increases in lung eosinophilia, high serum IgE levels, elevated IL-5 and IL-13 production, and increased mast cell numbers in the lungs (217, 219). Future work with this model may elucidate the mechanisms through which altered microbiota evokes the enhancement of allergic airway responses.

With the focus shifted to the importance of the healthy intestinal microbiota, there has been a substantial effort to assess the effects of probiotics on the prevention and/or treatment of allergic diseases in human clinical trials. In one such trial, *Lactobacillus* GG administered to high risk infants resulted in a 50% reduction in observed atopic eczema relative to the placebo group (140). Similarly, supplementation with *Lactobacillus reuteri* lowered the incidence of skin prick reactivity in infants with allergic mothers (2), while *Lactobacillus fermentum* reduced symptoms of atopic dermatitis in infants with moderate to severe disease (330). The PandA study demonstrated that a probiotic cocktail (*Bifidobacterium bifidum*, *Bifidobacterium lactis*, and *Lactococcus lactis*) was able to significantly reduce eczema in high-risk infants for a minimum of 2 yr provided that the probiotic was administered to the infant within 3 mo of birth (214). While these results indicate a strong correlation between probiotic supplementation and reduced atopy, not all epidemiological studies have reached the same conclusions, likely due to differences in the set-up of probiotic supplementation (150, 307). Probiotic administration in animal models of allergic asthma has yielded more convincing results (67, 73).

3. Type 1 diabetes

It is well established that type 1 diabetes (T1D) is an autoimmune disease, the symptoms of which are mediated by the self-reactive T-cell destruction of insulin-producing β-cells in the pancreas. What was not known until recently is that T1D is also linked to changes in the gastrointestinal microbiota. Dietary modifications that modulate the gut flora by reducing the total numbers of cecal bacteria were shown to be protective against T1D development in two rodent models [the Bio-Breeding diabetes-prone (BB-DP) rats (270) and the nonobese diabetic (NOD) mice (102)]. Fluorescent in situ hybridization (FISH) was used to characterize the microbiota of BB-DP

rats before and after the onset of diabetes. Healthy BB-DP rats that proceeded to develop diabetes had significantly higher numbers of Bacteroidetes relative to those rats that did not succumb to the disease. Interestingly, BB-DP rats that received a combination of antibiotics in addition to a standard diet showed a nearly 50% reduction in T1D incidence, while the diabetes incidence in rats that received the antibiotics and a high-casein (HC) diet was reduced to zero (29). Similar findings were reported by another group, where the frequency of T1D was reduced from 75 to 20% in NOD mice given the antibiotic dioxycycline in addition to the standard chow (269). Interestingly, these data contradict the negative effects normally associated with antibiotic usage. Reduced insulitis and β-cell destruction were observed in NOD mice receiving probiotic VSL#3 (a cocktail of *Lactobacilli*, *Streptococci*, and *Bifidobacteria*) three times a week; this corresponded to increased IL-10 production both locally (in PPs) and systemically (in spleen and pancreas) (33). While recent work with MyD88-negative ($MyD88^{KO}$) NOD mice has revealed that β-cell destruction in T1D is a MyD88-independent process (329), MyD88 contributes to T1D etiology through its impact on commensal flora. SPF $MyD88^{KO}$ NOD mice are protected from T1D while GF $MyD88^{KO}$ NOD mice are highly susceptible to the disease. The protection is restored when GF $MyD88^{KO}$ NOD mice are colonized with altered Schaedler flora (ASF). Similarly, T1D-associated islet cell infiltration is reduced in GF NOD mice cohoused with SPF $MyD88^{KO}$ NOD mothers. To determine how MyD88-dependent innate immunity influences the composition of the gut microbiota, 16S rRNA sequencing was performed on cecal contents from $MyD88^{KO/+}$ NOD mice and $MyD88^{KO}$ NOD mice. In contrast to data presented by Brugman et al. (29), Wen et al. (329) report that a significantly higher Bacteroidetes-to-Firmicutes ratio is present in the protected $MyD88^{KO}$ NOD mice (329). To explain this discrepancy, the authors argue that genetic changes in diabetes susceptibility might be mediated through different avenues than changes in susceptibility associated with dietary alterations. Despite the seemingly contradictory data published by these two groups, each of these studies has established that there is an important link between the gut microbiota and the development of autoimmunity, whether it is through dietary or immunomodulatory means.

4. *Familial Mediterranean fever*

Familial Mediterranean fever (FMF) is the first genetic disease to be linked to changes in the normal gut microbiota, providing evidence that host genotype plays a role in dictating the establishment and composition of the intestinal flora. FMF is caused by mutations in the *MEVF* gene, which encodes pyrin, an important regulator of innate immunity. After mapping mutations in the *MEVF* gene in control and FMF patients, FISH and 16S sequencing techniques were used to assess the effects of wild-type or mutated alleles of *MEVF* on the composition of the gastrointestinal microbiota. Differences in gut bacterial diversity were observed between FMF active patients, FMF remission patients, and healthy controls. The microbial diversity was significantly reduced in FMF active patients, which was likely a result of the inflammation associated with symptomatic disease. Interestingly, the microbial diversity associated with the FMF patients who were in remission was substantially greater than that observed in healthy controls. Statistical analysis revealed that the FMF remission gut flora clustered in a group that remained distinct from that of the controls, indicating that host genotype could play a role in defining these differences in community composition (146). Alternatively, these observations could indicate that recolonization of the microbiota following FMF-induced inflammation might proceed differently than colonization in a healthy, noninflamed individual. In a subsequent study, systemic IgG antibody titers against commensal bacteria were measured in the same three groups (FMF-active, remission and control) to determine whether the functionality of pyrin affects the translocation of commensal bacteria and their antigens beyond the gut epithelium. The level of systemic reactivity against antigens belonging to a number of commensal bacteria was significantly enhanced in both FMF-active and remission patients, suggesting that the functionality of pyrin affects the ability of commensals to breach the gut barrier, resulting in characteristically high systemic reactivity towards these bacteria in both active and remission states of FMF (190).

5. *Autism*

Very little is known about the underlying etiology of autism. Extensive antibiotic use is commonly associated with late-onset autism (18–24 mo of age), causing some to hypothesize that disruptions in the normal microbiota may allow colonization by autism-triggering microorganism(s), or promote the overgrowth of neurotoxin-producing bacteria like *Clostridium tetani* (24). The link between the intestinal microbiota and autism is supported by the following observations: *1*) disease onset often follows antimicrobial therapy, *2*) gastrointestinal abnormalities are often present at the onset of autism and frequently persist, and *3*) autistic symptoms have sometimes been reduced by oral vancomycin treatment, while relapse occurs following cessation of treatment (71, 259). These observations have been supported by qPCR (279) and culture-based (71) microbiota profiling techniques, which indicate that certain clusters of *Clostridium spp.* are present at 10-fold higher numbers in stool samples from autistic children compared with healthy controls.

Furthermore, the authors suggest that it is not a mere coincidence that exposure to trimethoprim/sulfamethoxazole antibiotics is much more likely to precede diagnosis of late-onset autism than exposure to any other antibiotic regimen. Trimethoprim/sulfamethoxazole antibiotics are not effective against *Clostridium spp.*, suggesting that early exposure to these drugs may promote an overgrowth of *Clostridium spp.* that could contribute to the etiology of autism (71). Interestingly, oral vancomycin specifically targets Gm+ organisms, among them *Clostridium spp.* Clostridia spores that remain viable after vancomycin treatment are believed to be responsible for the relapses that occur in autistic patients after discontinuation of vancomycin. One group has even suggested that Clostridia spores are the reason why high rates of autism are seen among siblings (70). Finegold et al. (71) suggested a number of mechanisms whereby the gut microbiota could be responsible for the debilitation of regressive autism including neurotoxin production by a subset of abnormal flora, autoantibody production that results in the attack on neuron-associated proteins, or microbial production of toxic metabolites that have neurological side effects (71).

F. Bacterial Translocation and Disease

In some circumstances, members of the gut microbiota migrate beyond their tightly regulated borders, and this disruption can cause systemic complications, promoting an entirely new repertoire of diseases targeting remote organ systems. The systemic presence of the intestinal microbiota and resulting associated complications can occur by two mechanisms. The first mechanism of systemic bacterial translocation relies on an internal cause such as impaired microvilli function, which leads to bacterial overgrowth and disruption of gut homeostasis resulting in the induction of initial systemic complications and disease onset (Fig. 8*A*). The second mechanism relies on an external injury or inflammatory reaction to cause stress on the body, leading to changes in intestinal permeability that promote bacterial translocation that results in further systemic complications and more serious disease outcomes (Fig. 8*B*).

1. *Bacterial translocation as the root of systemic complications*

Small intestine bacterial overgrowth (SIBO) is a condition in which colonic bacteria translocate into the small bowel due to impaired microvilli function, which causes a breakdown in intestinal motility and gut homeostasis (225). SIBO has been shown to positively associate with several diseases, including acute pancreatitis and fibromyalgia.

A) FIBROMYALGIA. The initial link between SIBO and fibromyalgia, a musculoskeletal syndrome, was provided by Whitehead et al. (333), who found that between 30 and 75% of affected patients exhibited symptoms of somatic hypersensitivity, which is also common among IBS patients (34). The lactulose breath test, which measures the hydrogen and/or methane evolved by bacterial metabolism and is used to diagnose SIBO, was shown to be positive in all 42 fibromyalgia patients enrolled in a study performed by Pimental et al. (237). The levels of hydrogen detected by the test were shown to correlate with the degree of somatic pain experienced by the patient. These same abnormal hydrogen/methane levels found in fibromyalgia patients were also detected in 84% of IBS patients (237). The positive SIBO test and the symptoms shared with IBS patients indicate that somatic hypersensitivity of fibromyalgia is likely influenced by the gut microbiota.

B) PANCREATITIS. Similar conclusions were made by Van Felius et al. (318), who were able to establish a link between the extent of SIBO and the severity of acute pancreatitis. Interestingly, a PCR screen of patient sera was able to detect DNA from Gm− bacteria in ~20% of pancreatitis patients, further substantiating a role for translocated gut bacteria in this disease (55). However, clinical trials testing the effectiveness of antibiotics as prophylactic treatment for pancreatitis-associated complications have had conflicting results. This indicates that elimination of gut microbiota from systemic sites is insufficient to reverse or stop the disease process, and more work needs to be done to elucidate the extent of the contribution of indigenous gut microbes to pancreatitis (17, 196).

2. *Bacterial translocation as accessory to systemic pathophysiology*

In some circumstances, outside stresses on the host (such as injury, infection, or unhealthy diet) can promote a dysregulation of intestinal mucosal homeostasis, resulting in a "leaky gut" syndrome and translocation of gut bacteria or bacterial products to systemic sites, which further aggravate the host's health.

A) TYPE 2 DIABETES. Specific microbiota community profiles have been suggested to promote metabolic disorders such as type 2 diabetes (T2D). Mice fed a high-fat (HF) diet, which promotes endotoxemia and enhances inflammatory tone, were shown to be more susceptible to T2D. qPCR analysis of cecal contents indicated that mice fed HF diets showed reduced levels of *Bifidobacteria* compared with control animals fed the standard chow (35). Prebiotic-mediated enhancement of *Bifidobacteria* in mice challenged with the HF diet reduced levels of endotoxemia proportionately to the observed increase in *Bifidobacteria*. Glucose tolerance and glucose-mediated insulin secretion also improved significantly with

increased numbers of *Bifidobacteria* detected in the gut (36).

B) ATHEROSCLEROSIS. HF feeding and the associated endotoxemia and vascular inflammation have also been shown to influence the onset of cardiovascular diseases, such as atherosclerosis. Recent evidence provided by Bjorkbacka et al. (22) suggests that a TLR4-dependent mechanism may be involved in the development of atherosclerosis. In this study, mice deficient in MyD88 (which, as mentioned previously, contributes to gut microbiota homeostasis) demonstrated a marked reduction in the early symptoms of atherosclerosis relative to wild-type controls (22). Furthermore, synbiotic supplementation was shown to significantly reduce the levels of LDL and fibrinogen (which promote atherosclerosis) in male patients with moderate cholesterol levels, suggesting that microbiota manipulation can be used to enhance cardiovascular health (31).

C) SYSTEMIC INFLAMMATORY RESPONSE SYNDROME AND BURN INJURY. The trauma associated with systemic inflammatory response syndrome (SIRS) has been linked to the gastrointestinal microbiota in a number of animal and human case studies. SIRS is an umbrella term that is used to describe the serious inflammatory state that arises as a result of traumas, infectious or otherwise. In one study, the fecal flora of 25 SIRS patients (18 sepsis, 6 trauma, 1 burn injury) was analyzed using a culture-based approach. The SIRS patients had significantly lower anaerobic bacterial counts, fewer "beneficial" *Bifidobacteria* and *Lactobacilli*, and correspondingly higher "pathogenic" *Staphylococci* and *Pseudomonas* compared with the healthy controls (273).

Several groups have focused their research specifically on the contributions of the gut microbiota to the associated side effects of burn injury. Animal models have been used to correlate thermal injury with intestinal epithelial apoptosis resulting in bacterial translocation (183, 184), the mechanism of which is currently unknown [although gut hypoprofusion was eliminated as a possible mechanism (241)]. Similarly, studies in humans have identified that the extent of burn injury correlates with the degree of intestinal permeability, or "leaky gut" syndrome (160, 253). Analysis of the intestinal flora in rats with thermal injury reveals significantly lower *Bifidobacteria* counts relative to Gm− organisms. Interestingly, rats that received an oral *Bifidobacteria* supplement demonstrated reduced intestinal damage and decreased bacterial translocation after only 3 days of probiotic therapy (328). Culture-based methods have established that translocation involves the movement of gut commensals from the intestine to the MLNs in response to thermal injury (184). MLNs have high vascular permeability, so it is hypothesized that bacteria from the gut travel to the MLNs where they can breach the endothelial barrier easily, enter the bloodstream, and cause systemic effects (185). This hypothesis is supported by evidence from Gonzalez et al. (93) who showed that MLN ligation prevents acute lung injury in posthemorrhagic rats.

G. Concluding Remarks

As the gut microbiota appears to contribute to nearly every aspect of the host's growth and development, it is not surprising that a tremendous array of diseases and dysfunctions have been associated with an imbalance in either composition, numbers, or habitat of the gut microbiota.

For certain conditions, such as enteric infections or IBD, the involvement of the indigenous microbial community is nearly intuitive, and many aspects of its contributions to the disease progression have already been conclusively demonstrated. However, many more questions still remain to be answered and fuel ongoing research in these areas, seeking to tease out the many subtle dysregulations in the interactions between the host and the microbiota and how they impact on the disease process.

For many other conditions, such as atopy or mood disorders, the possibility of microbial contribution is less apparent. Research looking into the involvement of the gut microbiota in these conditions is in its infancy, requiring further substantiation and elaboration. It is a poorly explored field full of exciting ideas and questions, answers to which hold promise to expand our knowledge of human pathophysiology.

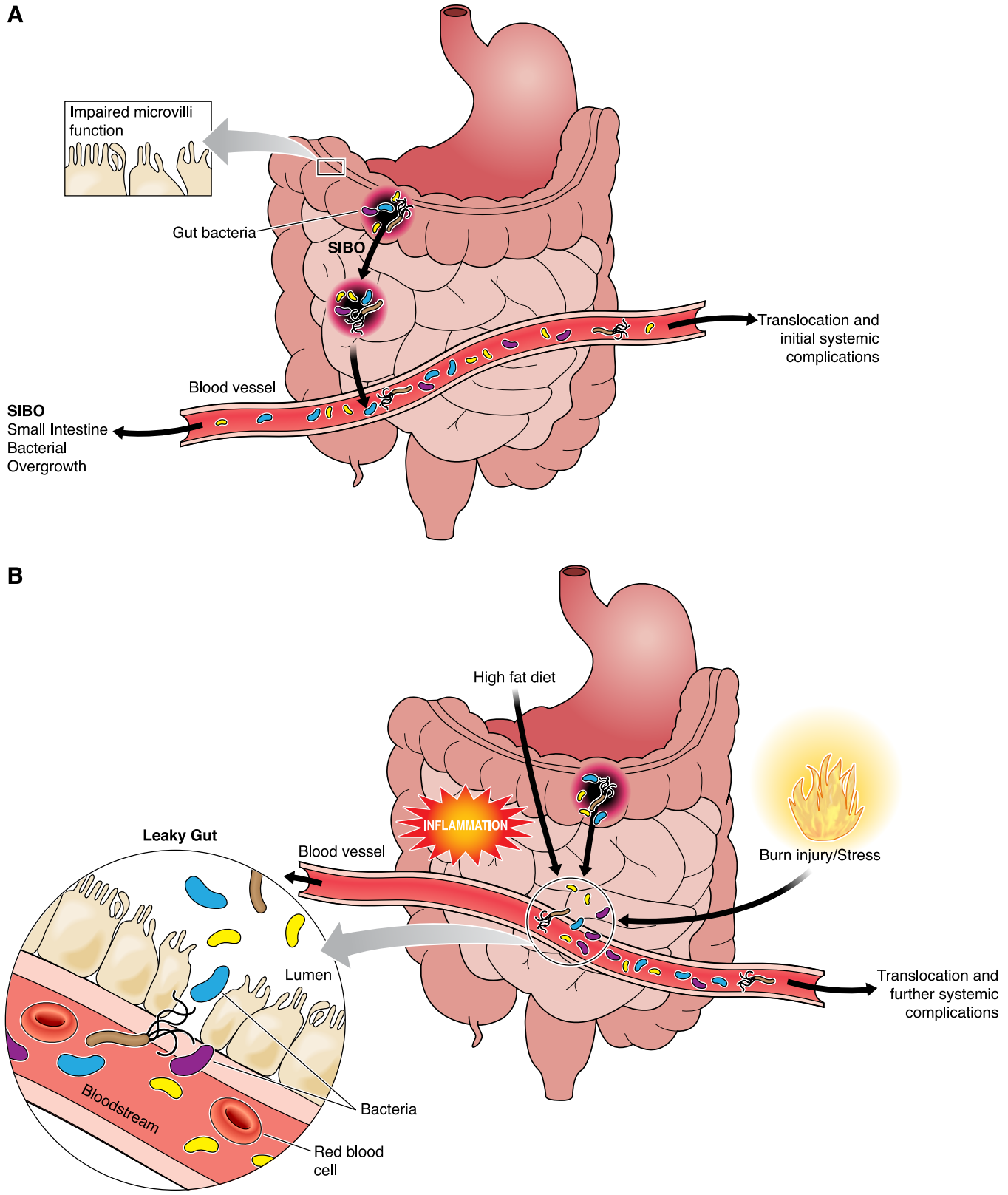
V. SIGNALING IN THE MAMMALIAN GUT

Communication between the host and its microbiota is necessary to put into effect the contributions of the gut bacterial community to both healthy and anomalous host processes. Therefore, understanding the communication, or signaling, pathways involved in these interactions is essential to enhance our knowledge of human physiology and our ability to correct or prevent pathophysiological processes.

Bacteria have been shown to produce and detect a myriad of extracellular signaling molecules. The mammalian intestine is an extremely complex and rich ecosystem and, as such, it provides an extensive platform for multiple layers of intercellular signaling between the components of the microbiota, the host, and incoming pathogens to occur (Fig. 9). Although this field of research is still in its infancy, a few examples of such interactions have been recently reported.

A. Signaling Between the Microbiota and the Host

Perhaps one of the first examples of intercellular signaling in the intestinal environment came from studies


A
Impaired microvilli function
Gut bacteria
SIBO
Translocation and initial systemic complications
Blood vessel
SIBO
Small Intestine Bacterial Overgrowth
B
High fat diet
INFLAMMATION
Burn injury/Stress
Leaky Gut
Blood vessel
Lumen
Translocation and further systemic complications
Bloodstream
Bacteria
Red blood cell

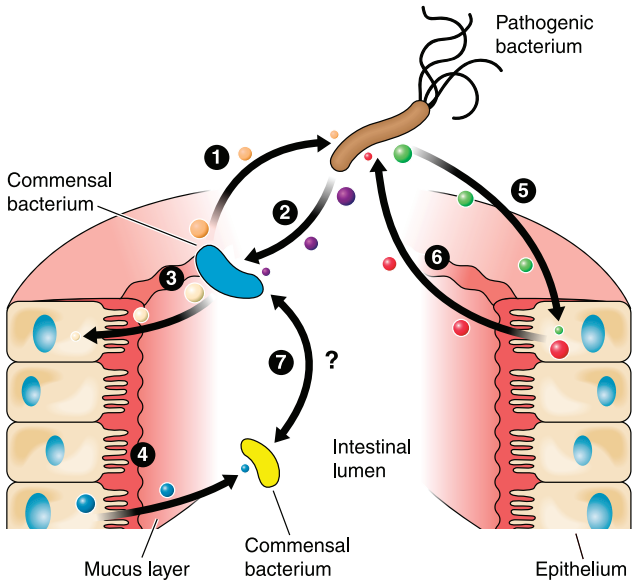


FIG. 9. Signaling between the intestinal microbiota, the host. and incoming pathogens. *1*) Molecules produced by the microbiota can have effects on pathogens. For instance, autoinducer 3 (AI-3) produced by the intestinal microbiota can activate enterohemorrhagic *Escherichia coli* (EHEC) virulence. *2*) Pathogens can also produce signaling molecules that may affect the intestinal microbiota. Pathogen-produced AI-3 may affect commensal gene expression. *3*) Signals produced by commensals can affect host cells. For example, *B. thetaiotaomicron* can produce a diffusible signal that induces host epithelium surface fucosylation. *4*) Host-produced molecules can affect commensals. Host-produced norepinephrine can impact the composition of the enteric microbial community. *5*) Pathogens can produce molecules that can be sensed by the host. For example, bacterial acyl-homoserine lactones have been shown to possess immunomodulatory activities. *6*) Host cells can produce molecules sensed by pathogens. One example is EHEC sensing of host-produced epinephrine and norepinephrine. *7*) Different species of commensals can interact with each other. For instance, colonization of mice with *B. thetaitaomicron* and *M. smithii* caused an increase in the bacterial density achieved when compared with colonization by each species alone. The question mark indicates that it is currently not known whether these interactions are of purely metabolic nature or are achieved through the production of bona fide signal molecules.

examining the impact of *Bacteroides thetaiotaomicron* on host physiology in the late 1990s. While studying the effects of colonization of germ-free mice with a conventional microbiota as well as *B. thetaiotaomicron* alone, Bry et al. (30) realized that the addition of certain sugars to the surface of intestinal epithelial cells could be controlled by this symbiotic bacterium. More specifically, *B. thetaiotaomicron* could induce the addition of fucose to the termini of surface carbohydrates of the epithelial cells. This phenomenon was regulated through an elegant mechanism that allowed *B. thetaiotaomicron* to induce host epithelium fucosylation only when fucose was not present in the intestinal environment (122). Under these circumstances, the fucose utilization pathway was shut off. As soon as the epithelium was coated with fucose moieties, *B. thetaiotaomicron* induced the expression of the genes encoding for enzymes involved in fucose degradation. At the same time, the induction of host fucosylation was abrogated. By doing so, *B. thetaiotaomicron* was able to tightly control the levels of fucose on the surface of the intestinal epithelium to maximize its growth, using fucose as a substrate, whilst avoiding the expenditure of inducing the host to produce more fucosylated surface glycans. Interestingly, binding of *B. thetaiotaomicron* to the intestinal epithelium was not required for the induction of fucosylation of host cells, suggesting that a diffusible molecule is involved (30). Although the molecule responsible for this phenomenon was never identified, this seminal study by Hooper et al. (122) has shown that signaling in the intestine can play important roles in the establishment and maintenance of the symbiotic relationship between intestinal microbes and their host.

Host immunomodulation by *B. fragilis* PSA molecule, which was described above, can also be considered from the point of view of microbiota-host signaling. PSA can be viewed as an example of a bacterial signaling molecule, eliciting a specific host response, namely, the upregulation of IL-10 secretion (197, 198).

Besides secreting molecules that can be sensed by their host, the intestinal microbiota can also sense host-produced molecules. Mammalian hormones are primarily involved in transmitting information from one site of the body to another. Several of these molecules are present in the intestine, suggesting that they may also affect the bacterial cells present in that environment (65, 142). An investigation on the impact of one of these molecules on the intestinal microbiota has been performed. Lyte et al. (177) induced the release of the catecholamine norepinephrine into the intestinal lumen by the administration of the selective noradrenergic neurotoxic agent 6-hydroxydopamine (6-OHDA) and followed the effect of such treatment on the growth of indigenous enteric bacteria (177). 6-OHDA administration caused a >1,000-fold increase in the numbers of aerobic and facultative anaerobic bacteria in the intestinal contents as judged by in vitro cultivation. Analysis of the bacteria recovered indicated

FIG. 8. Gut microbiota systemic translocation and associated diseases. *A*: microbiota translocation occurs as the result of an internal cause, such as impaired microvilli function. Damaged microvilli limit intestinal motility in the gut, promoting the translocation of bacteria from the colon to the small intestine, causing small intestine bacterial overgrowth (SIBO). SIBO facilitates the entrance of some of the displaced microbiota members into the bloodstream by breaching the endothelial barrier, causing complications outside of the gastrointestinal tract. *B*: external injury (e.g., burn injury or inflammation introduced from a high-fat diet) causes stress on the body, resulting in gut microbiota translocation due to a "leaky gut" (see *inset*). Translocation and resultant endotoxemia promote further systemic complications and increase the probability of damage to remote organ systems.

that the effect was due to an increase in growth of indigenous *E. coli* strains. Additionally, this effect was shown to be directly related to the release of norepinephrine caused by 6-OHDA given that treatment with the catecholamine uptake blocker desipramine hydrochloride abolished the effect of 6-OHDA on the composition of the intestinal microbiota (177).

B. Signaling Between the Microbiota and Pathogens

In addition to signaling to host cells, the intestinal microbiota can also communicate with incoming pathogens. The first indication that this was true came from studies of intercellular signaling in enterohemorrhagic *E. coli* (EHEC). EHEC is part of a group of intestinal pathogens called attaching and effacing pathogens (AE), due to the characteristic lesions that these pathogens induce in the intestinal epithelium upon infection. Central to the production of AE lesions is the type three secretion system encoded by the locus of enterocyte effacement (LEE) (40). Studies of intercellular signaling in EHEC have shown that the expression of LEE is controlled by quorum sensing (282). Later, it was shown that a molecule of unknown structure termed autoinducer 3 (AI-3) can activate the expression of the LEE and multiple other EHEC genes, some of which also have roles in virulence (283–285). Although EHEC can produce AI-3 and use it to control the expression of virulence, commensal bacteria present in the mammalian gut can also produce this signal. Sperandio et al. (284) have shown that cell-free supernatants of cultures of intestinal commensals can activate the expression of the LEE. This finding brought about the hypothesis that incoming pathogens could sense signals produced by the microbiota and take advantage of this by inducing the expression of virulence factors. Although this hypothesis was formulated around another quorum sensing molecule and AI-3 had not been discovered at that time, this rationale can be applied to any signaling molecule present in the intestine (282). EHEC has an extremely low infectious dose, estimated to be between 1 and 100 cells (158). Because EHEC can sense molecules produced by intestinal commensals to induce virulence, it has been hypothesized that the ability to sense AI-3 and/or other signaling molecules could be responsible for its low infectious dose (282, 284).

Another interesting study of intercellular signaling between the intestinal microbiota and incoming pathogens has been recently published and also involves EHEC sensing of commensal-produced molecules. In this study, de Sablet et al. (56) have shown that diffusible molecules produced by the intestinal bacterium *B. thetaiotaomicron* have the capacity to inhibit the production of Shiga toxin by EHEC. This is not due to AI-3, since supernatants of *B. thetaiotaomicron* cultures showed no detectable AI-3 activity. Moreover, de Sablet et al. (56) showed that this activity is not related to canonical quorum sensing molecules of *E. coli*, since mutations in multiple genes involved in EHEC quorum sensing did not affect the repression of *stx2* synthesis by *B. thetaiotaomicron* supernatants (56). Although some preliminary characterization of the signaling molecule involved has been performed, indicating that the signal is smaller than 3 kDa, the chemical nature of the molecule remains unknown.

Although it has been clearly shown that the intestinal microbiota can produce signal molecules that are sensed by incoming pathogens, the opposite is not true, i.e., the effect of pathogen-produced signals on the microbiota remains elusive. But, as previously mentioned, many of the signal molecules produced by bacteria are conserved between pathogens and commensals. It follows then that some of the molecules that are produced by intestinal commensals and that are sensed by incoming pathogens are likely to be produced by the pathogens themselves and can have effects on the physiology of multiple members of the resident gut microbiota.

C. Signaling Between Members of the Microbiota

Signaling between members of the intestinal microbiota can have important roles in the establishment and maintenance of homeostasis in the intestinal ecosystem. Samuel et al. (256) have recently studied the interactions between two prominent members of the human intestinal microbiota, the bacterium *B. thetaitaomicron* and the archaeon *Methanobrevibacter smithii*. Cocultivation of these organisms in the mouse intestine resulted in a >100-fold increase in colonization levels compared with colonization during mono-associations (256). Transcriptome analyses of these organisms grown in vivo showed that cocultivation has a profound impact on global gene expression of *B. thetaiotaomicron*. Specifically, cocultivation caused changes in multiple genes involved in carbohydrate metabolism (256). More recently, a similar study was carried out where the response of *B. thetaiotaomicron* to cocultivation with *Eubacterium rectale* was analyzed (186). As with the study of *B. thetaiotaomicron* interactions with *M. smithii*, this study showed that carbohydrate utilization systems of *B. thetaiotaomicron* are affected by cocultivation in the murine intestine (186). The interactions between *B. thetaiotaomicron* and these two members of the intestinal microbiota seemed to be specific since cocultivation of *B. thetaiotaomicron* with *Desulfovibrio piger* resulted in minor differences in the global transcription and metabolism of the former species (256). The mechanisms through which these interactions between *B. thetaiotaomicron*, *M. smithii*, and *E. rectale*

occur are still largely unknown. Although it is possible that these interactions are of metabolic nature and may not reflect bacterial communication per se, these studies demonstrate that intricate relationships between members of the microbiota can have significant effects in the gut ecosystem. Future studies in the field should try to elucidate these mechanisms, as well as ultimately progress to analysis of interactions in model systems comprised of hundreds to thousands of bacterial species, which would help further approximate the interactions in a fully colonized host.

D. Signaling Between the Host and Pathogens

In addition to interactions involving the intestinal microbiota and the host that occur in the gut, multiple levels of signaling also occur between the host and incoming pathogens. As previously mentioned, eukaryotic hormones can have an impact on bacterial populations in the gut. Specifically, the impact of the mammalian hormones epinephrine (EPI) and norepinephrine (NE) on the pathogenesis of EHEC has been intensely studied (46, 284). As with EHEC-produced AI-3, host-produced EPI and NE can also induce the expression of the LEE. This is accomplished through the newly identified bacterial adrenergic receptors QseC and QseE (46, 244). NE has also been shown to affect virulence of *Vibrio parahaemolyticus*, also by affecting the expression of one of its type three secretion systems (209). Additionally, NE can affect the growth of multiple pathogenic *Vibrio* species (208). Another example of a host molecule that has an effect on bacterial virulence is the opioid hormone dynorphin. This hormone is present in the GIT and has been shown to activate one of the quorum sensing systems of *Pseudomonas aeruginosa* and, consequently, virulence gene expression (345).

Although studies on the communication between the host and pathogens have focused on bacterial sensing of host molecules, host cells can also sense bacterial signals. It has been known for quite some time that the *P. aeruginosa* quorum sensing molecule 3-oxododecanoyl-homoserine lactone (3OC12-HSL) has immunomodulatory activities (308). 3OC12-HSL can enter mammalian cells (248), and it has been recently shown to disrupt NFκB signaling (152), showing that bacterial signals can have profound effects on host cells.

VI. MODELS TO STUDY MICROBIOTA

A. Germ-Free Animals

Over the past several decades, a number of animal models have been used to study the dynamic, ecologically diverse community of microbes that reside in the GIT and to help us understand the biological complexities of the processes that govern host-microbiota symbiosis. The intestinal microbiota has been studied in a variety of animal models, including gnotobiotic and GF mice (30), pigs (203), and zebrafish (243). When used under a defined set of parameters, these simplified model ecosystems can provide us with insights about how the colonization status of the host affects vital host processes. In addition, these models are able to better facilitate the study of individual members of the microbiota so that unique roles for different gut microbes can be established and put in the context of different health and disease perspectives.

A lot has been learned about the role of the intestinal microbiota in human health and disease from research using GF animals. GF animals enable researchers to explore how host functions are affected by colonization with commensal microbes or lack thereof.

As mentioned earlier, GF animals exhibit pronounced defects in multiple aspects of their organs' structure and function (see Table 1 for summary). Despite these caveats, GF animals are a wonderful research tool, the use of which has provided the foundation for much of the ground-breaking work that has been done to elucidate the mechanism behind the existence of this complex microbial community. GF models are advantageous because they offer a simplified experimental system in which diseases or specific members of the gut microbiota can be studied in isolation. Some disease models have been evaluated in GF animals alongside their conventional counterparts, and the results have revealed startling differences that have implicated the gastrointestinal microbiota in the etiology of numerous diseases, such as IBD (25), graft-versus-host disease (GVHD) (109), and allergy (106).

GF models have also been used for studying the effects of "probiotics" on the host. For instance, this type of screening was used by Kabir et al. to recognize that the probiotic strain *Lactobacillus salivarious* inhibits the attachment and colonization of *Helicobacter pylori* (138). However, while these models can be used to screen for candidate probiotic therapies, the successful candidate strains need to be tested in more biologically relevant (i.e., conventional) animal models before they can be considered for use as therapeutics.

The use of GF animals does, however, have some drawbacks. As gut microbiota is crucial for the proper development of the host, altered responses exhibited by GF animals might not reflect what actually occurs in the natural setting (66). Thus it may be challenging to transfer results obtained in a GF system to the same series of events occurring in a conventional host.

TABLE 1. *Germ-free animal model studies*

Observations in Germ-Free Animals	Possible Mechanism	Potential Implications for Science/Medicine	Arising Research Questions
Increased longevity	ND	The use of pre/pro/synbiotics in geriatric populations	Is the increased longevity due to less inflammation-mediated damage in germ-free animals?
		Extension of end points in basic geriatric research	Is the increased longevity due to absence of detrimental metabolites produced by the normal gut microbiota?
Increased radioresistance	ND	Further defining the pathophysiological mechanisms underlying radiation-induced tissue damage	What is the contribution of microbial metabolites to radiation-induced tissue damage?
		Improvements to tissue damage in radiotherapy	What is the contribution of microbiota-stimulated intestinal epithelial renewal to radiation-induced tissue damage?
Increased adipose deposits in geriatric animals	ND	Further defining the role of microbiota in obesity	Does microbial-host signaling promote generation of adipose deposits?
			Can pre/pro/synbiotics be used therapeutically in metabolic syndrome and insulin resistance?
Decreased intestinal surface area	Reduced villous thickness	Further defining the mechanisms of nutrient absorption	Which microbial metabolites are involved in promoting anatomical maturation of the GI tract? What is the time frame of their activity?
	Reduced crypt cell production Reduced villous capillary network	Studies of signaling in postnatal anatomical development	Can pre/pro/synbiotics be used therapeutically in GI mucosa atrophy?
Impaired bile acids and cholesterol metabolism	Largely ND	Potential for pro-/pre-/synbiotic therapeutic or prophylactic interventions in atherosclerotic disease and hypercholesterolemia	Which bacterial species/products are involved in maintenance of bile acid metabolism?
	Impaired cholesterol metabolism likely secondary to impaired bile acids metabolism		Which signaling pathways are targeted?
Impaired local and systemic lymphoid organ structure	ND	Enhanced understanding of prenatal lymphoid organogenesis	What are the relative contributions of different bacterial species/products to lymphoid organogenesis?
		Studies of signaling in postnatal lymphoid development	Does local and systemic lymphoid organ maturation happen simultaneously or sequentially?
Reduced levels of secretory immunoglobulins	Mechanisms per se ND	Enhanced understanding of signaling involved in isotype switching	Which component(s) of segmented filamentous bacteria is involved in IgA induction?
	Segmented filamentous bacteria stimulate IgA production	Potential improvements to vaccine responses	
Impaired generation of oral tolerance	Reduced number of CD25+CD4+ Treg cells	Enhanced understanding of food allergy and tolerance	Which bacterial species/products induce CD25+CD4+ Treg cell expansion?
	Reduced production of TGF-β by CD25+CD4+ Treg cells	Potential improvements to vaccine responses	Can CD25+CD4+ Treg cell expansion and activity be induced independently of each other?
Decreased cardiac output	ND	Potential implications for treatment of disorders resulting in decreased cardiac output (e.g., dilated cardiomyopathies)	Is decreased cardiac output due to a reduced stroke volume, reduced heart rate, or a combination of both factors?
		Enhanced understanding of cardiac physiology	Is the abnormality largely anatomical or physiological? Is it reversible?
			Is the presence of live bacteria/microbial components or the presence of microbial metabolites required to normalize cardiac output?

TABLE 1—*Continued*

Observations in Germ-Free Animals	Possible Mechanism	Potential Implications for Science/Medicine	Arising Research Questions
Hypotonic and hyporesponsive mesenteric vasculature	Exact mechanisms ND	Potential implications for treatment of hypertension	Is the abnormal lumen-to-wall ratio of germ-free arterioles the "default" state that can be corrected by conventionalization, or does it develop over time in the face of abnormal physiology of germ-free animals?
	Proposed mechanisms: *1*) greater lumen:wall ratio of mesenteric arterioles *2)* accumulation of compounds that antagonize vasoactive substances in the cecum	Enhanced understanding of determinants of vasculature development	Would modifications in microbiota numbers/composition skew the host towards hypo- or hypertonic responses?
Exaggerated stress response of HPA axis	Possible pathways:	Uncovering the etiology of gastrointestinal disorders with a neurogenic component (e.g., irritable bowel syndrome)	Which microbial species/products are involved in the brain-gut axis signaling?
	Microbe-mediated release of cytokines (e.g., IL-1) that stimulate secretory activity of HPA axis	Further defining the mechanisms of neural development	Which neural pathways are affected, both centrally and peripherally?
Decreased sensitivity to inflammatory pain	SCFAs mediated release of 5-HT from enterochromaffin cells that stimulates 5-HT_3 receptors on vagal afferent fibers Direct production of neuroactive substances by the microbiota Increased production of IL-10 Reduced bioactivity of inflammatory mediators	Potential therapeutic implications for the treatment of neurogenic disorders	What is the relative contribution of the brain-to-gut versus gut-to-brain signaling in the development of associated gastrointestinal and neuropsychiatric disorders?

HPA, hypothalamic-pituitary-adrenal; TGF, transforming growth factor; 5-HT, 5-hydroxytryptamine; IL, interleukin; ND, not defined.

B. Mono-Associated and Bi-Associated Animals

Gnotobiology is the colonization of GF animals with select microbial species/strains. Studies involving animals associated with just one or two commensal species allow the investigation of host-microbe interactions in a simplified ecosystem. Mono-associated animal models provide information about that particular microbe's ecological niche, what it provides the host with, and how the host responds to this microbe in the absence of competition from other microbes. Similarly, studies of bi-associated animals can reveal how two microbes interact with one another and with the host, and whether cocolonization changes the functional roles they establish in the gut ecosystem as a result of competition for space and nutrients. Evidence from mono- and bi-associated models of human feces-derived *Eubacterium rectale* and *Bacteroides thetaiotaomicron* by Mahowald et al. (186) indicate that gene expression patterns change in both organisms from mono-association to cocolonization, and niche specialization occurs where the metabolic requirements of both organisms are reciprocated rather than competed for. This investigation, as well as others by the same group (256), demonstrate the ability of gut microbes to adapt to their surroundings and carve out a very specific ecological niche when they are influenced by neighboring bacteria. Because the gut microbiome changes depending on the consortium of its members and sequence in which colonization by these members occurs, mono-associated and bi-associated microbiota models are powerful tools that enable us to observe how members of the microbiota change their metabolic needs in response to cocolonization. While these models do provide unique insights into the niches occupied by particular members of the gut microbiota, they are also complicated by the fact that these bacteria respond to cocolonization in species- and sequence-dependent manners. For instance, in a human infant, the facultative anaerobes colonize the GIT first, followed by the obligate anaerobes. Interactions between different commensal microbes following artificial colonization of GF animals that happens in a different sequence might not reflect the interactions in a natural state. Consequently, studying individual species in isolation may provide insights into what ecological niche they may occupy; however, any given organism will need to be observed in its natural ecosystem before final conclusions are drawn.

C. Poly-Associated Animals

The concept of poly-associated animal models was introduced in the mid-1960s by Russell W. Schaedler

(266). The goal was to selectively colonize GF mice with eight defined bacterial strains (isolated from conventional mice), achieving a standardized gut flora that would serve as a powerful research tool. The Schaedler flora was revised in 1978 and became known as the altered Schaedler flora (ASF), which is the standardized poly-associated flora "cocktail" used in laboratories today. Recent 16S rRNA profiling has revealed that ASF consists of two *Lactobacillus spp.*, a relative of *Bacteroides distasonis*, a spiral-shaped bacterium belonging to the *Flexistripes* phylum, and four fusiform extremely oxygen-sensitive (EOS) bacteria (59). What becomes more important than the actual identities of the strains of bacteria contained within the ASF is the ability of this set of standardized flora to reflect the gut ecosystem in a CV animal. Half of the bacterial strains represented in the ASF are EOS bacteria belonging to the Firmicutes phylum. Several recent surveys of the gut microenvironment have revealed that EOS bacteria represent the majority of the members of the GI microbiota in mice and rats and that these bacteria outnumber facultative anaerobes by 100:1 and aerobes by at least 1,000:1 (96, 104, 263). Two members of the ASF are *Lactobacillus spp.*, which are an aerotolerant subset of the Firmicutes and commonly colonize the stomach and small intestine of humans and other CV mammals (61, 249). One of the two remaining strains (*Bacteroides distasonis*) belongs to the Bacteroidetes phylum, the second most dominant phylum represented in the flora of CV animals. The last bacterial strain present in the ASF cocktail is a spirochete from the *Flexistipes* phylum, which has been found in significantly large numbers in the ceca and colons of mice (250) but remains less well-characterized in the flora of other mammals.

ASF animal models are considered advantageous because they are colonized with a defined set of intestinal flora. Because the flora in CV animals varies significantly between different animal facilities and even between cages housed within the same facility, colonizing animals with the standardized ASF flora better facilitates cross-study comparisons because variation introduced by differences in the gut ecosystems of different research animals can be eliminated. Also, ASF rodents do not have to be bred GF because offspring can inherit the ASF from colonized mothers. Embryo transfer has been used to effectively colonize new mouse strains (e.g., knockout strains, transgenics, etc.) with ASF. Recent 16S rRNA sequencing of the gut flora in mice indicates that the ASF remains stable (i.e., free of contaminating bacteria) in a mouse colony over a long term (292). These stability tests do not eliminate the need to actively monitor the composition of the ASF, because undetected shifts in the flora could significantly alter the outcome of subsequent experiments. ASF animal models are advantageous because they utilize a standardized gut flora, but an intestinal microbiota consisting of eight bacterial species is significantly limited in its ability to mimic the complexity of the normal intestinal microbiota, which is currently estimated to contain anywhere between 800 and >1,000 bacterial species (11). While the ASF does do an adequate job of representing the dominant phyla present in a CV animal, the community dynamics in a natural gut ecosystem cannot be reproduced by such a small number of organisms; therefore, the ASF cannot be expected to demonstrate completely comparable host-microbiome interactions.

D. Human Flora-Associated Animals

Comparative studies using 16S rRNA sequencing have demonstrated that the intestinal microbiota in mice and humans is very similar in composition at the division level (i.e., ≥80% of both mouse and human microbiotas are dominated by two phyla, the Firmicutes and the Bacteroidetes). However, individual species differ significantly between them (64). In an attempt to circumvent this difference, human flora-associated (HFA) animals are produced by inoculating GF mice with fecal suspensions from human donors. Whether these ex-GF mice behave like conventional mice is still under debate.

HFA animals can be used as models to investigate how the human intestinal flora interacts with the host, as well as how dietary changes and therapeutics (e.g., probiotics, prebiotics, antibiotics) impact host gut ecology and metabolism (89). Studies with HFA animals have revealed that the role of human fecal flora is somewhat different from the role of the normal flora in other animals (112), suggesting that HFA models could serve as a useful tool to render studies involving the intestinal microbiota more applicable to humans. Studying the role of the human gut flora in animal models rather than in human subjects is advantageous because the variability introduced by differences in genetic and environmental factors can be eliminated. HFA models also are useful in studies where it is unethical to use human volunteers, such as studies involving colonization resistance with pathogenic organisms or investigating the toxic effects of chemicals or carcinogens (112).

While HFA animals appear to be the closest non-human equivalent for investigating the role of the human intestinal microbiota, they do have their limitations. Recent metabolomic analyses have revealed that the metabolic profiles of HFA animals differ from those obtained from human subjects, with some metabolites differing more significantly than others. Hiriyama et al. (111) found that putrefactive products and SCFA levels were lower in the feces of HFA mice compared with humans. However, the composition of these products was more similar to those found in humans than in mice (111). These discrepancies can be rationalized by the fact that different

hosts maintain different relationships with their commensal microbes. Therefore, transferring the microbiota from one host to another (e.g., from a human to a mouse) will not necessarily produce a functionally equivalent intestinal environment because of differences in the metabolic needs of the new host. Another important consideration concerning the use of HFA animals is the composition and stability of the corresponding intestinal flora. According to Hirayama et al. (114), once established, the human intestinal flora remains stable over a long period of time and can be passed on to subsequent offspring in a manner that resembles CV mice; however, the sequence of colonization events that occur in the human infant could not be reproduced in the HFA pups (114). Also, upon comparing the composition of the HFA flora in different mice, it was noted that while their dominant phyla remained consistent, *Bifidobacteria spp.* were absent in some (113).

All animal models have their limitations. HFA animals offer the most applicable system for studying the ecology and metabolism of the human intestinal flora, and as long as the limitations are known, the appropriate conclusions can be drawn and made applicable to the human case.

VII. TECHNIQUES TO STUDY MICROBIOTA DIVERSITY

A variety of techniques (summarized in Table 2) are available to study gut microbial communities. Their respective advantages and limitations are discussed below.

A. Culture-Based Analysis

Classically, the composition of the microbiota has been analyzed using culture techniques that use differential media to select for specific populations of bacteria based on their metabolic requirements. Culture-based techniques of bacterial enumeration are cost-effective and reproducible. However, they are limited in their ability to distinguish between different bacterial phylogenetic groups. Species- or strain-level detection becomes incredibly difficult, if not impossible. Studying the complex microbial community in the gut is especially difficult because the majority of these bacteria are strict anaerobes, and it is estimated that >80% of the gut microbiota cannot be cultivated under standard laboratory conditions (64).

B. Culture-Independent Techniques

Since the observation that culture-based methods of microbiota analysis were inadequate, microbiologists have turned to the molecular-based techniques traditionally used in microbial ecology to characterize complex marine and soil communities (90, 137). These techniques use the bacterial 16S ribosomal RNA (rRNA) gene as a marker of genetic diversity. The 16S rRNA gene was chosen because of its relatively small size (~1.5 kb) and the fact that it strikes an appropriate balance of conservation and variability with enough variation present to distinguish between different species and strains, yet enough similarity to identify members belonging to the same larger phylogenetic group (233). The community profiling techniques that take advantage of the 16S rRNA gene come in many forms, each of them offering their own benefits and drawbacks.

C. Sequencing Methods

1. Full-length 16S rRNA sequencing

The diversity of the gastrointestinal microbiota was first thoroughly characterized in three healthy subjects by Eckburg et al. (64) using a full-length 16S rRNA sequencing method (i.e., Sanger sequencing). To determine the extent of the bacterial diversity, 16S rRNA sequences are "binned" into operational taxonomic units (OTUs) based on their percent sequence identity (%ID). Specific %IDs are widely accepted as indicators of various tiers of taxonomic resolution. OTUs containing sequences with ≥99% pairwise sequence similarity indicate "strain-level" taxa, while ≥97% ID designates "species," ≥95% ID "genus," and ≥90% ID "family" (233).

2. Pyrosequencing

Cost is the most significant limitation of the Sanger sequencing method of microbial community analysis. As sample numbers increase, so does the budget required to perform the analysis. Newer, more cost-effective options are now replacing the need to sequence each 16S rRNA gene in its entirety. Pyrosequencing generates large numbers of 16S rDNA sequence tags by amplifying select variable regions within the 16S rRNA gene. It is capable of sequencing 25 million bases at 99% or better accuracy in one 4-h run, achieving 100-fold higher throughput than Sanger sequencing (192). The shorter sequence reads require only targeted amplification of select highly variable regions of the 16S rRNA gene (e.g., V2, V3, or V6 regions are typical regions of interest) so that higher taxonomic resolution can be achieved using smaller sequence reads (100). Often, multiple variable regions are sequenced so that what was a class-level taxonomic identification (e.g., variable region V2 has class-level resolution) can be narrowed down to a genus or species (298). Pyrosequencing is practical because it eliminates the time-consuming step of creating clone libraries (discussed below) and employs the use of bar-coded primers, which allow multiple samples to be mixed in a batch sequencing run. The bar-code

TABLE 2. *Gut microbiota analysis techniques*

Technique	16S rRNA Based?	Cost ($)	Taxonomic Resolution/ Sensitivity	Advantages	Disadvantages (Limitations)
Culture based	No	$	Moderate	You have the organism "in hand"	Most GI organisms cannot be cultured in current defined media
				Functional information gained from what is known about the organism's substrate utilization and other physiological parameters	Labor intensive
Full-length (Sanger) sequencing	Yes	$$$$	Very good	Sequencing the entire 16S gene maximizes the taxonomic resolution offered by the gene	Expensive (although mechanization is reducing the cost)
					Extensive bioinformatic analysis required
454 Pyrosequencing	Yes	$$$	Good to very good	Higher throughput than Sanger sequencing (200,000 sequences versus 20,000)	Can't obtain the taxonomic resolution that can be achieved with full-length sequencing
				More sensitive (can detect less abundant organisms due to the number of reads obtained)	Shorter sequence reads (<500 bp versus 1.5 kb)
				Multiple samples can be analyzed in a single sequencing run	Extensive bioinformatic analysis required
				No cloning bias introduced	
				Less susceptible to PCR bias (shorter PCR amplicons, less influenced by G/C content)	
DGGE	Yes	$	Poor	Rapid	Shorter PCR products mean less taxonomic information can be obtained
				Fingerprints provide a good basis to compare communities from different treatment groups	Reproducibility between gels is difficult
				Bands of interest can be excised and sequenced	
TRFLP	Yes	$$	Poor	Fingerprints provide a good basis to compare communities	Limited taxonomic resolution
				Multiple restriction enzymes can be used to provide greater resolution	One "phylotype" can represent more than one species
				Reproducible	Capillary sequencer required
RISA	No	$$	Good	Greater variability between species and strains than the 16S gene	Limited phylogenetic data currently available; no extensive RIS database developed for gut bacteria
				When a better database has been developed, taxonomic resolution could be "Excellent"	A single bacterium can have more than one different RIS region
DNA microarrays	Yes	$$$$	Very good	Incredibly useful as a screening approach	Detection limited by the sequences contained on the chip (no detection of uncharacterized phylotypes)
				Fast, easy to use	Cross-hybridization issues
				Clinical applications	
FISH	Yes	$$$	Good	Can target specific bacterial groups/species of interest (they must be preselected)	Can't identify novel groups of bacteria
				Flexible scope: probes can be designed to target groups or individual species	It's not a community-wide survey of "who's there"
				Direct enumeration of bacteria-16S copy number is not an issue	Reference strains are required to validate the results
					Microscope work is time-consuming (however FACS options are becoming available)

TABLE 2—*Continued*

Technique	16S rRNA Based?	Cost ($)	Taxonomic Resolution/ Sensitivity	Advantages	Disadvantages (Limitations)
qPCR	Yes	$$	Good	Can target specific bacterial groups/species of interest (they must be preselected)	Reference strains are required to validate the results
				Flexible scope: primers can be designed to target groups or individual species	Can't identify novel groups of bacteria
					It's not a community-wide survey of "who's there"
					16S copy number varies between 1 and 10
Metagenomics	Genome wide	$$$$$	Good	Provides a community-wide assessment of the functional genes present	Shotgun reads are mapped to reference genomes; this is limited by the number of sequenced genomes available
				16S gene sequences provide taxonomic identification of community members	Extensive bioinformatic analysis required
				Sensitivity depends on the number of sequence reads obtained	Cloning biases could affect the functional gene information obtained
					No direct information about which genes are expressed or functioning
Metabolomics	No	$$$$	Poor	Metabolic profiles can be used to compare communities in a functional context	No taxonomic information available
				More direct functional information can be obtained	The source of each metabolite is unknown; therefore, it is difficult to identify what organisms are producing what compound
				More rapid and less expensive than metagenomics	Not all metabolites are detectable with current technology
				Nontargeted approach can also identify host metabolites associated with the gut microbiota	
Metaproteomics	No	$$$$	Poor	Metaproteomes can be used to compare communities in a functional context	No taxonomic information available
				More direct functional information can be obtained	Protein abundance is difficult to estimate
				More rapid and less expensive than metagenomics	Less abundant proteins (from populations making up <1% of the community) go undetected
				Nontargeted approach can also identify host proteins associated with the gut microbiota	
Metatranscriptomics	Genome wide	$$$$$	Good	Provides insights into community-wide structure and function	RNA is much more easily degraded than DNA; this could cause information loss
				Can be used to detect changes in community-wide gene expression profiles in response to different environmental stimuli	Sensitivity of community analysis depends on the number of sequence reads (via pyrosequencing) obtained
				No biases introduced by PCR or cloning steps (none required)	
				Transcripts can be measured quantitatively	

A number of techniques have been applied to analyze the composition, abundance, and function of the gastrointestinal microbiota over the last several decades, 16S rRNA-based and otherwise. The analysis method of choice depends on the question being asked as well as the time and cost restrictions associated with the task. An assessment of the benefits and limitations of the current techniques available in gut microbial ecology is provided. GI, gastrointestinal. RIS, ribosomal intergenic spacer.

approach allows specific sequences to be traced back to the samples they came from. New error-correcting bar codes are able to run hundreds of samples at once (100).

The recent examples of pyrosequencing use are comparisons of gut communities between obese and lean twin pairs (312), as well as assessment of microbiota perturbations and stability during and following the use of antibiotics (58).

D. "Fingerprinting" Methods

Pyrosequencing and full-length 16S rRNA sequencing technologies are massive undertakings that generate thousands of sequences and require extensive data analysis. There are a variety of other community profiling tools used by microbial ecologists that are capable of answering similar questions with reduced expense and effort. DNA fingerprinting is a community analysis tool that generates a DNA profile of the microbial community in each sample, and thus allows comparison between samples based on the differences observed between their genetic "fingerprints."

1. Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a technique that denatures the PCR-amplified gene of interest (e.g., 16S rRNA) from the extracted community DNA. The PCR-amplified gene products obtained from each community sample will migrate on an acrylamide gel according to their G+C content (the most stable DNA migrating further) creating a distinct banding pattern that represents the amount of diversity in the sample (i.e., more bands on a gel are indicative of greater diversity). DGGE analysis is typically performed on PCR amplicons that are small in size (~150 bp) and highlight only regions of the gene that demonstrate the greatest variability (e.g., V6-V8 region of the 16S rRNA gene). DGGE patterns can be quantified by measuring the number and thickness of bands on the gel. Statistical tools like principal coordinate analysis (PCA) can be used to measure differences between DGGE patterns so as to detect changes from one condition to another. This approach was used by Zoetendal et al. (351) to identify differences in the mucosa-associated bacterial communities in the colon and feces of patients with UC. DGGE PCR amplicons are too small to get enough sequencing information to correctly identify the bands of interest. Therefore, this technique is used primarily for comparative purposes.

2. Terminal restriction fragment length polymorphisms

Unlike DGGE, terminal restriction fragment length polymorphisms (TRFLP) use full-length 16S rRNA PCR amplicons obtained from the isolated community DNA. TRFLP profiles are generated by digesting the PCR-amplified 16S rRNA gene products with a restriction endonuclease, which gives rise to a terminal restriction fragment (fluorescently tagged so that it can be detected by a capillary sequencer) that varies in length depending on the particular sequence of the 16S gene (224). The different fragment lengths migrate differently on a gel, creating a distinct banding pattern for each sample. Variations between communities can be found in the size and number of bands in the profile, and individual bands can be traced back to individual organisms with the aid of a clone library. As is the case with all fingerprinting techniques, one band on the gel should represent one phylogenetic type ("phylotype"), or theoretical "species." However, comigrating bands containing PCR products with the same terminal restriction fragment (TRF) will appear as a single phylotype. Repeating the process with different restriction endonucleases will create a different series of TRFs and allow differentiation between these bands. TRFLP is a method that is both rapid and reproducible and has been optimized to measure variability within the human gut microbiota (164).

3. Ribosomal intergenic spacer analysis

Ribosomal intergenic spacer analysis (RISA) is a technique that is relatively new to the field of gut ecology, yet it is a tool commonly used by microbial ecologists to characterize the complex bacterial communities in marine and soil environments (86, 277). RISA involves PCR amplification of the intergenic spacer region between the 16S and 23S rRNA genes. The 5′ primer is labeled with a fluorescent tag, which can be detected by a capillary sequencer, producing a fingerprint of ribosomal intergenic spacer (RIS) fragments created by the extent of length heterogeneity in the sample. The RIS is a hypervariable region because it lacks the evolutionary pressure forced upon the rRNA genes. Because of its enhanced variability, RISA could eliminate the problems associated with species-level identification experienced with alternative 16S rRNA sequencing methods. The enhanced variability of the RIS could resolve differences between species or strains that remain indistinguishable using other methods (80). The internal transcribed spacer (ITS), as it is referred to in eukaryotes, is widely used to taxonomically characterize plants and fungi (42, 148). Several recent studies have used RISA to identify the enhanced colonization of certain bacterial species in patients with diseases like colorectal cancer (265) and IBD (151) compared with healthy controls.

While the RIS does offer the advantage of higher resolution when it comes to complex community analysis, there are several drawbacks to the technique that have yet to be addressed. Because RISA has been restricted to soil

and marine environments until recently, there is no extensive database containing RIS taxonomy for gut microorganisms like the 16S rRNA databases. A significant amount of work needs to go into these databases before RISA can be a universal tool for in-depth analysis of the intestinal microbiota. This technique is complicated further by the fact that bacteria have multiple RIS regions of varying lengths (81, 98), which means that bacterial identification is not as simple as matching one RIS fragment to a particular species.

Although "fingerprinting" methods of gut bacterial community analysis have many great advantages, one notable drawback is their high limit of detection. The detection limits for DGGE, TRFLP, and RISA are much higher than those obtained with Sanger or pyrosequencing methods because these fingerprinting techniques are limited by the ability of the amplified fragments to be resolved on a gel. A phylotype that is present at a lower abundance than 1% of the total membership for that community will not be differentiated from the background noise detected by the fragment analysis software.

E. DNA Microarrays

DNA microarray technology has been one of the most challenging tools to develop in the field of microbial ecology. It is designed specifically for high-throughput screening of human gut microbial communities and, as such, is a very powerful tool. The first extensive DNA microarrays containing probes designed to detect members of the gastrointestinal microbiota were developed in the Brown laboratory (228, 229). These arrays were based on an Agilent platform containing probes targeting up to 359 microbiota species (229), as well as up to 316 "novel OTUs" (228) identified during studies of human colon (64) and stomach (20) microbial ecology. More recently, an even more sensitive microarray representing 775 phylospecies was developed by Paliy et al. (227). They used an Affymetrix GeneChip platform containing 775 representative sequences of phylospecies clusters obtained from human feces and several colon sites. The Affymetrix platform was chosen because of its increased sensitivity compared with other platforms (227). In comparative studies, this chip was able to detect and quantify differences in the gut microbiota of healthy adults and children and was able to detect bacterial DNA present at 0.00025% of the total community DNA (227).

A gut-specific phylogenetic microarray offers numerous benefits to both clinical and biomedical research fields; it is a cost-effective and less time-consuming alternative to 16S sequencing methods, which provides similar levels of sensitivity, selectivity, and quantification. Currently, issues surrounding detection limits and hybridization biases (i.e., some sequences hybridize more readily than others) still persist and need to be addressed before such gut microbiota microarray chips can be marketed commercially.

F. FISH and qPCR

In addition to the large-scale high-throughput screening methods presented above, there are two other tools that can be used to screen the intestinal microbiota. These techniques utilize fewer probes to target distinct groups of bacteria and are useful when specific bacterial phylogenetic groups are being targeted.

FISH uses fluorescently labeled oligonucleotide probes designed to hybridize to 16S rRNA sequences unique to the targeted bacterial groups. Depending on the probe design, FISH can be used to target large groups of bacteria (i.e., phylum-level classification) or it can be more specific (i.e., genus or species-level classification). Recently, a set of 15 FISH probes has been designed that can identify ~90% of the normal intestinal microbiota (103). FISH has been used to broadly characterize phylum-level shifts in the gastrointestinal microbiota that occur in response to enteric infection (175, 272), and it has also been used by clinical researchers as a tool to compare the gut flora composition between diseased (e.g., atopic, IBS-prone) and healthy individuals (139, 145).

Another technique that can be used to target specific bacterial groups in complex mixtures is qPCR. As with FISH, primers for qPCR can be designed to be as specific or as general as needed. However, sometimes primers designed to amplify one bacterial group also amplify members of other closely related groups. FISH and qPCR techniques are often used in combination to confirm the observed results (145).

The disadvantage of using microarray, FISH, or qPCR methods of microbial analysis is that since the chips, probes, and primers are designed to look at specific bacterial taxonomic groups, it is not possible to identify novel species/strains of bacteria. Additionally, interpreting qPCR results requires the use of a reference strain to generate a standard curve, which can be complicated when there is no suitable culturable strain available.

G. The "Meta" Family of Function-Focused Analyses

While surveying the composition and numbers of the gut bacterial communities promotes our knowledge of the identities of our microbial inhabitants, it does little to tell us about their function. Appreciation of the functional contributions of gut microbial communities to their host is essential to generate a complete view of the ecology and functional capacity of the gut microbiome.

1. Metagenomics

Metagenomics is one of the newest additions to the repertoire of microbial community analysis tools. It is a comprehensive approach that gives sequence information from the collective genomes of the microbiota, which can in turn be used to identify the functional contributions and biological roles of this complex community in human health and disease. This method is unique because it is not dependent on the cloning and sequencing of particular genes. Instead, it provides a detailed survey of all the genes that exist within a particular community, lending insight to both structure (composition) and function in a single experiment. Metagenomics involves shotgun sequencing, where reads of large cloned fragments isolated from total community DNA (a shotgun read is equivalent to the size of ~1 gene) are stitched together into contigs (>1,000 bp of contiguous sequence) that are then assembled into scaffolds (contigs + gaps) that approximate whole genomes. In the case of simple communities, it is possible to obtain complete genomes from the more abundant members within that community; however, as previously indicated, the gut microbiota is not an example of a simple community. The study of complex communities using the metagenomics approach rarely involves the onerous task of assembling whole genomes, due to the lack of sequence coverage obtained from a given sequencing run. The lack of whole genome sequence information available in the public databases also limits the ability to identify gene function based on known sequence information. Instead, metagenomic sequencing reads are entered directly into gene databases of interest whereby the origins of particular genes can be determined using information contained within those databases (314, 320).

Approximately 80% of a bacterial genome encodes proteins, making it highly likely that at least a partial gene relaying functional information is contained within each shotgun read (215). The depth of coverage depends on the number of sequences obtained and the number of contigs successfully assembled. The number and type of genes sampled from the community can be biased by the plasmid preparations of microbial DNA required for shotgun sequencing. Genes that are toxic to *E. coli* may not be included in the metagenomic data set because they could not be propagated in the host strain during cloning (281).

Once the genomes have been assembled, the protein-coding genes are identified using a BLASTp analysis, and these predicted gene products are given a COG (cluster of orthologous groups of proteins) assignment based on the reference database (306). The COG database provides predicted functional information for each gene. These gene products could be queried against a variety of other databases that cater to specific functions like KEGG (Kyoto Encyclopedia of Genes and Genomes) (141), CAZymes (Carbohydrate-Active enZymes), and STRING (functional protein association networks) (324).

Several groups have published data detailing the complexities of the human gut "microbiome" (89, 155). Gill et al. (89) were the first to compare the microbiomes of two healthy individuals and emphasize the validity of the "superorganism" concept. Kurokawa et al. (155) analyzed 13 intestinal microbiomes of adults, children, and infants, revealing that while the infant-type microbiomes show significant interindividual variation in gene composition and function, the microbiomes from adults and weaned children demonstrate high levels of functional redundancy, providing evidence that a "core microbiome" exists in the human gut. These observations were later confirmed by Turnbaugh et al. (312) in an obese and lean twin study, where the group was able to demonstrate that a "core" collection of genes is present among healthy individuals; however, the idea that a similar set of "core" microbial species exists to contribute these genes appears to be incorrect. Metagenomics was also used to conclude that deviations from a healthy body weight (i.e., obesity) correlated with the loss of several "core" functional components (312).

The metagenomics approach to gut microbial ecology allows assessment of the functional contributions provided by this symbiotic organ to its host. The next step would involve defining how these functional contributions are utilized or are affected by the host, and what role host genetics plays in this complex host-microbiota relationship (146, 190).

2. Metaproteomics

Whole community proteomics, or metaproteomics, is another function-based approach that has recently been used to identify key microbial functions in the gut. Metaproteomics utilizes non-targeted shotgun mass spectrometry to assess the diversity and abundance of proteins contained within the gut metaproteome. It was recently used to analyze the complex proteome of the human distal gut microbiota (321). Several comparisons have been made between the results of metaproteomic and metagenomic analysis of 2 separate sets of fecal human samples (89). The proteins identified in human fecal samples differed significantly from the distribution of proteins predicted using metagenomics, suggesting that functional gene analysis does not necessarily correlate well with gene expression levels (321). To confirm the accuracy of these observations, however, comparisons need to be made between matched datasets.

Besides being advantageous because microbial protein expression levels can be directly monitored, metaproteomics is useful because the protein separation steps can be altered so as to select for host proteins that interact with specific microbial proteins, providing in-

sights about the interactions required to maintain host-microbiota symbiosis. Of course, as this technique has just recently begun to be applied for analysis of a complex microbial community, significant optimization steps are still pending. Purification of microbial proteins needs to be improved, and detection capabilities enhanced, to facilitate the recognition of contributions from microbial community members present at lower abundance.

3. Metabolomics

Metabolomics is another technique used to study the function of complex microbial populations through survey of their metabolic profiles. It is a simultaneous analysis of multiple small metabolites present in a given sample. Some of the most advanced technologies allow for the identification of hundreds and sometimes thousands of metabolites in a short period of time (101). Recently, metabolomics has been used to characterize the impact of the murine intestinal microbiota on blood metabolites, showing that the intestinal microbiota has a profound and systemic impact on host metabolism (335). One limitation of metabolomics is that a comprehensive view of all the metabolites present in a sample is still not possible given the high complexity of most body fluids and tissues. Nevertheless, metabolomics has proven to be a powerful methodology that will certainly aid in identifying and characterizing the effects of the gut microbiota in health and disease.

4. Metatranscriptomics

Perhaps one of the newest "omics" technologies applied to the study of microbial communities is metatranscriptomics. This approach is similar to metagenomics in which it relies on the high-throughput sequencing of nucleic acids isolated directly from complex microbial populations. However, as the name implies, metatranscriptomics involves the characterization of the RNA content of samples, as opposed to the DNA content which is analyzed in metagenomics approaches. Metatranscriptomics has been successfully used to study complex microbial communities in soil (315) and aquatic environments (78), as well as the host-symbiont interactions in the termite gut (305). Akin to metagenomics approaches, metatranscriptomics can be used to generate structural information (i.e., community membership) while simultaneously obtaining functional insights about a microbial community. Because metatranscriptomics utilizes RNA transcripts rather than DNA, it can provide information about the dynamic nature of a community and help us draw conclusions about how changes in the surrounding environment induce community-wide alterations in gene expression. Although studies using metatranscriptomics to characterize the interactions between mammalian hosts and their microbiota remain to be seen, this technique has the undoubted power to aid in our current understanding of human-microbe interactions.

VIII. FUTURE PERSPECTIVES: HAVE WE GOT THE GUTS FOR IT?

Gut microbiota now appears to influence the host at nearly every level and in every organ system, highlighting our interdependence and coevolution. Its adaptation to our changing life-styles (such as diet- and ethnicity-associated differences in gut microbiota composition) is astounding, highlighting that the consequences of our behaviors affect not only the environment without, but also that within us.

Determining the details of the gut microbiome's involvement in our development, and function in both health and disease holds promise of enhancing many aspects of our daily lives, from optimizing the composition of infant formulas to offering new tools in our fight against pandemics of cancer and obesity. However, to reach this stage, research efforts must pose and answer concrete questions detailing specific aspects of host-microbe relations and the mechanisms underlying them. Improvements in the tools available to microbiota research, and especially the shift from "who's there" to "what do they do" type of approaches will certainly advance our knowledge of this area, although population complexity and diversity between individuals make this challenging. And while we try to regulate the numbers and composition of our gut microbiome, we need to thoroughly appreciate the many levels and areas of the host-microbiota interdependence and realize that this regulation is bidirectional.

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Address for reprint requests and other correspondence: B. B. Finlay, 301-2185 East Mall, Michael Smith Laboratories, Univ. of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada (e-mail: bfinlay@interchange.ubc.ca).

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