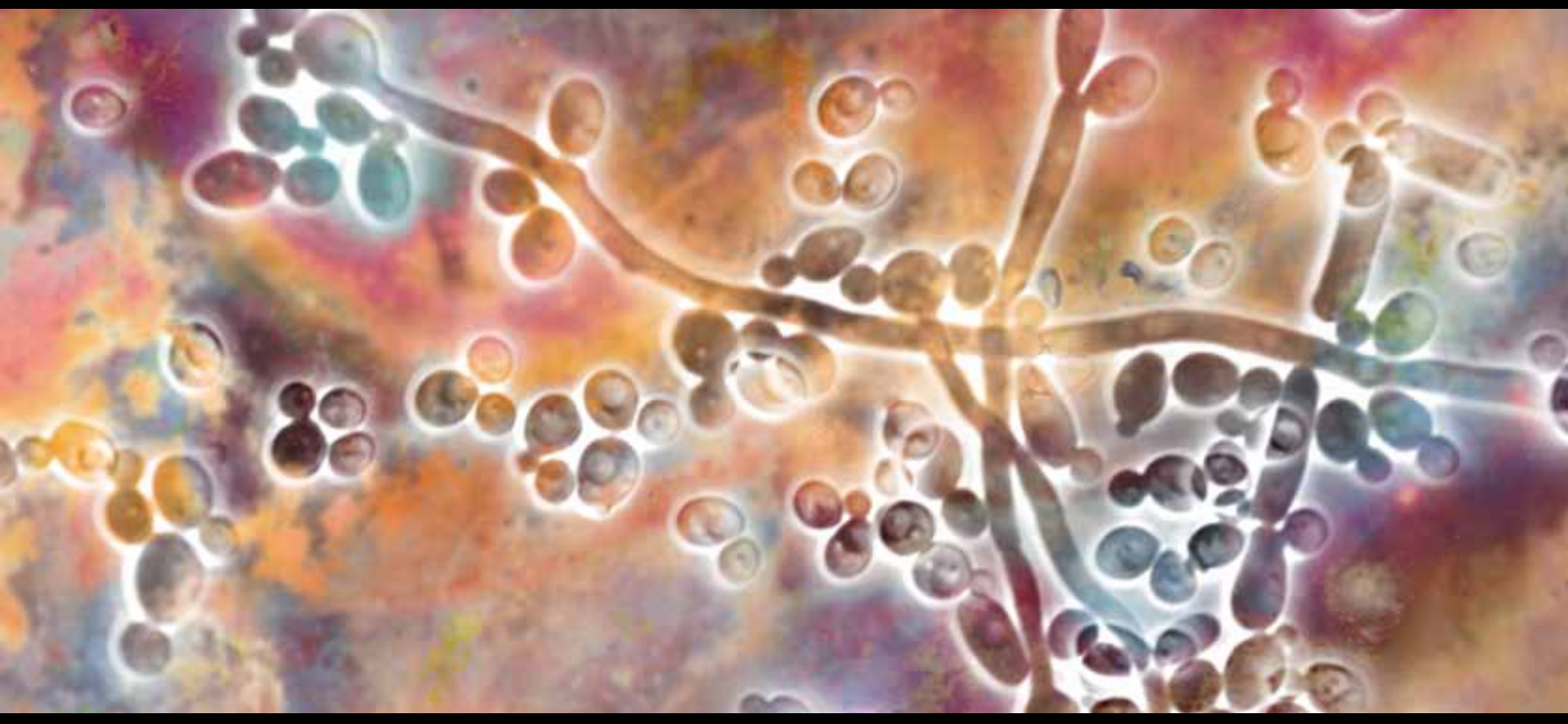
Interdisciplinary Perspectives on Infectious Diseases

The Human Microbiome and Infectious Diseases: Beyond Koch

Guest Editors: Vincent B. Young, Robert A. Britton, and Thomas M. Schmidt



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Editorial

The Human Microbiome and Infectious Diseases: Beyond Koch

Vincent B. Young,[1,2] Robert A. Britton,[3] and Thomas M. Schmidt[3]

[1] *Division of Infectious Disease, Department of Internal Medicine, University of Michigan, MI 48109, USA*
[2] *Department of Microbiology and Immunology, University of Michigan, MI 48109, USA*
[3] *Microbiology and Molecular Genetics, Michigan State University, MI 48824-4320, USA*

Correspondence should be addressed to Vincent B. Young, youngvi@umich.edu

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A century after Robert Koch linked individual cultured microbes to specific diseases (Koch's postulates), it has become increasingly apparent that the complex community of microorganisms associated with the human body plays a key role in health and disease. The National Institutes of Health recently announced the Human Microbiome Project as part of the NIH Roadmap for medical research, with a primary goal of advancing our understanding of the relationships among host-associated microbial communities, health, and disease. Many physicians and researchers, however, have only passing familiarity with the concepts involved in the study and therapeutic manipulation of complex microbial communities.

This special issue was conceived to accomplish several goals. We wanted to provide readily accessible overviews of the concepts and methods used in the study of complex microbial communities, and demonstrate how changes in indigenous microbial communities can play a role in diseases such as antibiotic-associated diarrhea and bacterial vaginosis. We also set out to find examples of how probiotics can be used for the therapeutic manipulation of the indigenous microbiota.

We were fortunate to receive a strong collection of review articles and primary research manuscripts to meet the goals of this special issue. In the first article "Conceptualizing the human microbiota: from multicelled organ to ecological community," B. Foxman et al. present a novel conceptualization of the human microbiome that blends perspectives of epidemiologists, classical ecologists and infectious diseases physicians. The second article "Application of ecological network theory to the human microbiome," by J. Foster et al., outlines how ecological network theory can be applied to studies of the human microbiome, while the third article "Interactions of the intestinal epithelium with the pathogen and the indigenous microbiota: a three way crosstalk," by C. V. Srikanth and B. McCormick, review the interactions between epithelial pathogens and the indigenous microbiota in the mammalian gut. In the fourth article "Application of sequence-dependent electrophoresis fingerprinting in exploring biodiversity and population dynamics of human intestinal microbiota: what can be revealed?" G. Huys et al. review the use of sequence-dependent fingerprinting methods for studying the structure and dynamics of complex microbial systems using the human intestinal microbiota as an example. The fifth article "Ecological characterization of the colonic microbiota of normal and diarrheic dogs," by J. Bell, employs such a fingerprinting method to study the canine colonic microbiota. The sixth article "Emerging insights into antibiotic-associated diarrhea and *Clostridium difficile* infection through the lens of microbial ecology," by S. Walk and V. Young, discusses the role of the gut microbiota in antibiotic-associated diarrhea and *Clostridium difficile* infection while Y. Sanz et al., in the seventh article "Insights into the roles of gut microbes in obesity," review the evidence for the role of gut microbes in obesity. In the eighth article "The human vaginal bacterial biota and bacterial vaginosis," by S. Srinivasan and D. Fredericks review the human vaginal bacterial microbiota and the ninth article "Temporal shifts in microbial communities in non-pregnant african-american women with and without bacterial vaginosis," by J. Wertz et al., examines this microbial community in the setting of bacterial vaginosis. The tenth article "Vaginal microbiota

and the use of probiotics," by S. Cribby et al. discusses the use of probiotics to alter the vaginal microbiota. Finally, the eleventh article "Probiotics and gastrointestinal infections," by R. Britton and J. Versalovic, and the twelveth article "Probiotic bacteria influence the composition and function of the intestinal microbiota," by P. O'Toole and J. Cooney, summarize the potential role of probiotics to influence gastrointestinal health.

ACKNOWLEDGMENTS

The editors hope that this collection of articles will be useful to a wide range of investigators who are interested in the role of the indigenous microbiota in health and disease. The editors wish to thank the authors who submitted their work for this special issue, and to the staff at Hindawi for aiding us in the preparation of this issue.

Vincent B. Young
Robert A. Britton
Thomas M. Schmidt

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Review Article

Conceptualizing Human Microbiota: From Multicelled Organ to Ecological Community

Betsy Foxman,[1] Deborah Goldberg,[2] Courtney Murdock,[3] Chuanwu Xi,[4] and Janet R. Gilsdorf[1,5]

[1] *Department of Epidemiology, University of Michigan, Ann Arbor, MI 48109, USA*
[2] *Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109, USA*
[3] *School of Natural Resources and Environment, University of Michigan, Ann Arbor, MI 48109, USA*
[4] *Department of Environment Health Sciences, University of Michigan, Ann Arbor, MI 48109, USA*
[5] *Department of Pediatrics, University of Michigan, Ann Arbor, MI 48109, USA*

Correspondence should be addressed to Betsy Foxman, bfoxman@umich.edu

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The microbiota of a typical, healthy human contains 10 times as many cells as the human body and incorporates bacteria, viruses, archea, protozoans, and fungi. This diverse microbiome (the collective genomes of the microbial symbionts that inhabit a human host) is essential for human functioning. We discuss the unstated assumptions and implications of current conceptualizations of human microbiota: (1) a single unit that interacts with the host and the external environment; a multicelled organ; (2) an assemblage of multiple taxa, but considered as a single unit in its interactions with the host; (3) an assemblage of multiple taxa, which each interacts with the host and the environment independently; and (4) a dynamic ecological community consisting of multiple taxa each potentially interacting with each other, the host, and the environment. Each conceptualization leads to different predictions, methodologies, and research strategies.

1. INTRODUCTION

The scientific community has just begun to appreciate the number and complexity of organisms inhabiting the human body. The human microbiota contains 10 times as many cells as the human body and incorporates bacteria, viruses, archea, protozoans, and fungi. Many essential body processes require the presence of these diverse microorganisms to maintain pH in the oral and vaginal cavities, prevent invasion by pathogenic organisms, stimulate the immune system, aid digestion, and provide nutrients essential to our health. If a diverse microbiota is essential for human functioning [1], disruption of the normal microbiota should have significant negative consequences for human health. Indeed, studies suggest that the gut microbiota can influence risk of obesity [2], inflammatory bowel disease [3], cardiovascular disease [4, 5], and allergies and asthma [6].

The National Institutes of Health recently launched a series of initiatives focused on characterizing the human microbiome, the collective genomes of the microbial symbionts that inhabit a human host. Characterizing the microbiome provides insight into the diversity of genomes inhabiting the human host and is a first step towards understanding the complicated interactions among symbionts and between the symbionts and the human host. This launch has stimulated much discussion on why and how the human microbiome should be characterized. There has been little explicit discussion, however, of the underlying conceptualizations or models of the microbiota which might guide this characterization. Models provide a framework for designing experiments and for making inferences and predictions. In this commentary, we describe the range of conceptualizations of the human microbiota that have been implicit in different segments of this emerging literature. By making explicit the underlying models, we reveal the underlying assumptions and can consider the strengths and weaknesses of the different models in fitting existing observations, identify important data gaps, make predictions, and consider what model best applies in a given situation or for a given research or clinical question.

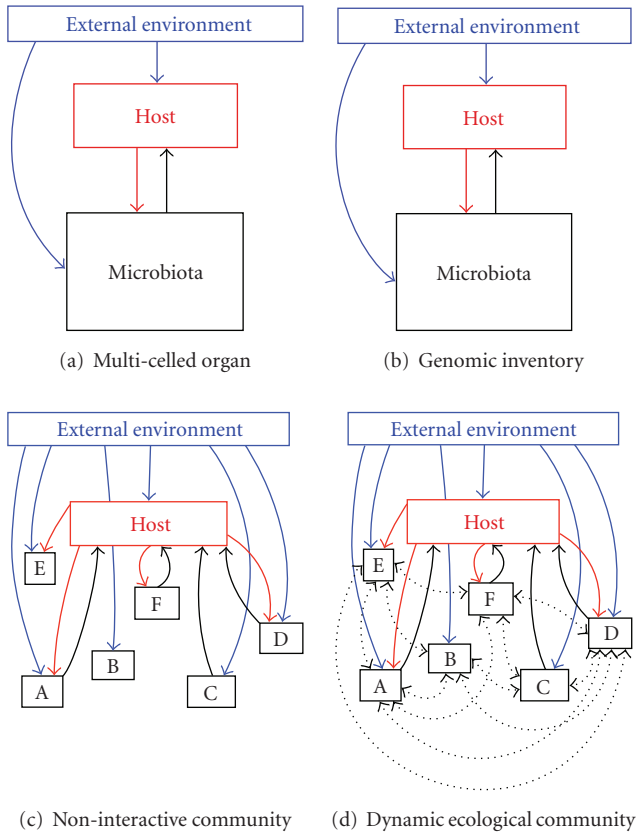

FIGURE 1: Four conceptualizations of human microbiota that focus to varying degrees on structure and/or function of the microbiota as a whole or of the component microbial taxa. Assumptions and implications of the extremes of simplicity and tractability on one hand (the multicelled organ conceptualization, Figure 1(a)) and complexity and relative intractability (the dynamic ecological community conceptualization, Figure 1(d)) are described in Table 1. All the interactions (linking arrows) are mediated to some extent by changes in the internal environment, which is not shown to enhance clarity. Mechanisms underlying the various interactions, including the role of internal environment, are depicted in Figure 2.

Figure 1 graphically displays the range of conceptualizations of the microbiota implicitly described in the following literature.

(1) The microbiota considered as a single unit that interacts with the host and the external environment; a multicelled organ.

(2) The microbiota consisting of multiple taxa, but considered as a single unit in its interactions with the host.

(3) The microbiota as an assemblage of multiple taxa, which each can interact with the host and the environment independently.

(4) The microbiota as a dynamic ecological community consisting of multiple taxa each potentially interacting with each other, the host, and the environment.

Conceptualizations (1) and (4) are clearly extremes and most research probably falls somewhere between them. However, because they are extremes, we can more clearly contrast them and their underlying assumptions, which have different implications for the development of clinical interventions (see Table 1). We expect an understanding of the human microbiota to require a melding of conceptualizations and associated theories before the promise of translating this understanding to new prevention, diagnostic, and treatment strategies can be achieved.

2. THE MICROBIOTA AS A MULTICELLED ORGAN

The microbiota is implicitly assumed to be much like a multicelled organ in much of the medical literature (Table 1) [2]: like an organ, a healthy microbiota consumes, stores, and redistributes energy and mediates important chemical transformations that benefit the host [7]. Communication among the cells that make up the microbiota enables replication and repair, and a set of feedback loops link host and microbiota (Figure 1(a)). The focus of an organ conceptualization is on *function*, with metabolic products and immune or neurological responses depending on the microbiota as a whole [7]. This view also implicitly assigns borders to the unit of interest, assuming that each spatially defined set of microbiota—the gut, oral community, or vaginal community—exists as a distinct and independent entity, and that each entity interacts with the host and the external environment as a single unit. Perhaps most importantly, this conceptualization assumes that any variation in the microbiota over time and between individual hosts is not functionally important or can be overlooked because of redundancies in genetic elements encoding various metabolic pathways in different strains or species. These unstated assumptions, summarized in Table 1, have the advantage of simplifying the system and focusing our attention on measuring inputs and outputs, physical structure, and defining spatial boundaries.

Conceptualizing the microbiota as an organ suggests research should characterize the range of inputs and outputs and immune response to the outputs and correlate them with healthy and diseased states for development of diagnostics. This conceptualization also implies that a therapeutic that adjusts the inputs and outputs could return the organ to a healthy state or substitute for a poorly functioning organ. For example, early diabetes—a malfunctioning pancreas—is diagnosed by measuring organ inputs (glucose levels), and is treated by decreasing inputs (lowering glucose levels) or supplying output (insulin). We might envision similar inputs and outputs that can be used to diagnose and correct disrupted microbiota in the skin, mouth, gut, or vaginal cavity.

Assuming a physical structure and boundaries stimulates studies to explore that structure and define boundaries. For example, conceptualizing the microbiota as an organ leads us to consider that the microbiota on the skin or intestinal lumen might form physical structures, such as biofilms. This structure might vary in size and composition, being a thick lawn in some areas and thin islands in others and act as an additional physical barrier to colonization by pathogens.

Table 1: Underlying assumptions of conceptualizing human microbiota as a multicelled organ versus an ecological community. Some of the assumptions of the multicelled organ conceptualization also apply to the intermediate conceptualizations depicted in Figure 1.

	Multicelled organ	Ecological community
Assumptions	(1) Identification of component microbes is not necessary for prediction of function	(1) Understanding interactions among microbiota is essential to predict function
	(2) Metabolic products and immune responses are characteristic of the microbiota as a whole	(2) Metabolic products and immune responses are a consequence of community structure and microbial interactions
	(3) Static (changes in healthy microbiota over time are not functionally important)	(3) Dynamic
	(4) Boundaries exist (movement of microbes is not important)	(4) Spatially continuous and linked by immigration and emigration
	(5) Host-to-host variation in microbiota is not important	(5) Host-to-host variation is functionally important
	(6) Microbiota functions for benefit of the host	(6) Net microbiota effects can range from negative to neutral to positive
Implications	(1) Healthy microbiota function is evaluated by its metabolic products and immune responses	(1) Healthy microbiota function is evaluated by both microbial community structure and its metabolic products and immune responses
	(2) Health is restored by providing the right signals/products that are missing or by neutralizing negative signals/products	(2) Health is restored by shifting the community and component interactions, which requires an understanding of processes that control community structure and interaction webs
	(3) Appropriate therapies include broad-spectrum antibiotics, microbiota transplants, direct manipulation of metabolic products, or immune signals	(3) Appropriate therapies include carefully tailored probiotics, modification of internal, or external environment to modify specific interactions

Disrupting these protective biofilms chemically or physically may lead to invasion by pathogens. Additionally, the size or denseness of the structure might in some surfaces be associated with disease. Assuming a defined boundary suggests that microbiota might be moved or be removed, and that there are optimal areas for measuring inputs and outputs. These are all testable hypotheses. The disadvantage of conceptualizing the microbiota as an organ is that it necessarily minimizes the complexity of a diverse microbiota, which may lead us to either underestimate the possible unintended consequences or overestimate the potential of proposed interventions.

The current research focus on cataloging the diversity of microbiota using genomic techniques [8] takes a step beyond viewing the microbiota as a single, homogeneous unit (Figure 1(b)). While a critical next step, this approach goes no further than the basic organ-view in understanding the *mechanisms* that drive variation in function of the microbiota; the underlying assumptions and implications of this approach remain quite similar to those of the "microbiota as organ" conceptualization (Table 1).

3. MICROBIOTA AS AN ECOLOGICAL COMMUNITY

The other extreme is to conceptualize the microbiota as a continuum of dynamic ecological communities living in the numerous microhabitats of the human body [9]. Each species or strain of the microbiota interacts with other members of the microbiota and with the host, as well as with the external environment (Figure 1(d)). This conceptualization highlights interactions between component organisms and their dynamics; a dynamic and spatially continuous system is assumed, and the net effects can be positive, negative, or neutral towards the host (Table 1). Key to this conceptualization is that understanding the underlying processes that control community structure, including the interactions among the microbiota themselves, is essential for understanding its function. This conceptualization has the advantage of increased realism, but is much more complex and consequently may be less useful for some purposes.

Considering microbiota as an ecological community stimulates research into how that community reacts to insults. For example, a number of conditions, such as reactive arthritis, occur in some individuals in response to infection. One current theory is that certain microbial surface antigens mimic host cell receptors, so individuals with a particular variant in immune signals generate an immune response to their own cells after infection has cleared. The role of microbiota in mediating this response has not yet been considered. However, we know that the gut microbiota is important in modulating host immune response [2]. It is possible that bacteria that lead to reactive arthritis disrupt the signals between the human body and the microbiota such that the immune system no longer sees organisms with antigens similar to those of the host as self, leading to self-attack. Consequently, the reason that reactive arthritis is frequently self-limiting may be related to restoration of the normal microbiota with subsequent restoration of immune signals.

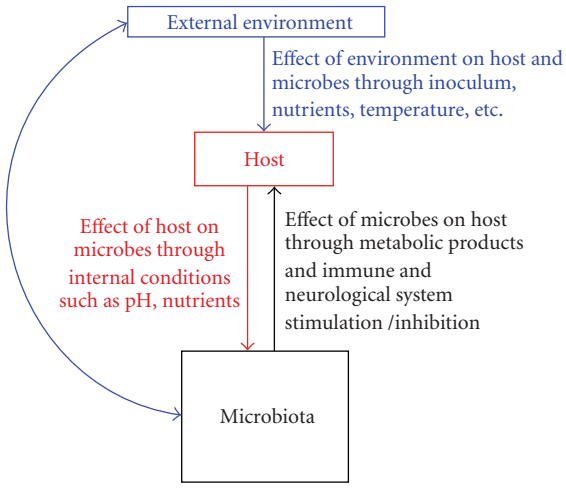

Figure 2: Potential mechanisms of interactions between external environment, host and the microbiota in the multicelled organ conceptualization of human microbiota.

While some research has conceptualized the human microbiota as an ecological community, the interactions among microbiota remain almost completely unexplored [10]. Most work is similar to the conceptualization in Figure 1(c), characterized by independent relationships between each member of the microbiota and its human host, but not among the microbiota themselves [5]. However, we suspect that interactions among members of a community, including the numerous indirect pathways of influence generated in such webs, are integral to understanding the dynamic and spatially heterogeneous nature of many aspects of the human microbiota and, therefore, to the functioning of those communities [11–13]. If so, however, complex and difficult, research must address how this understanding of ecological dynamics and function can be translated to successful clinical interventions.

4. RESEARCH AND CLINICAL CONSEQUENCES OF ALTERNATIVE CONCEPTUALIZATIONS OF THE HUMAN MICROBIOTA

The underlying conceptualization of the microbiota guides, either explicitly or implicitly, medical approaches to treating and preventing conditions of disrupted flora. An organ view assumes that switching from an unhealthy (dysfunctional) to a healthy (functional) state can be achieved by manipulating inputs or outputs. With this model in mind, the associated research agenda will focus on characterizing the products of the microbiota, their healthy and diseased ranges, and how the products are affected by host characteristics and the external environment (Figure 2). Therapeutic studies will seek to shift metabolic products or cell signals back to the functional state associated with health.

By contrast, if we conceptualize the microbiota as multiple communities of interacting genomes, we might instead try to reestablish or maintain a specific microbial community structure associated with health. Success of this approach depends on reestablishing a healthy microbial community, with all its associated feedbacks. The fact that we currently lack sufficient understanding to establish complex ecological communities with a full complement of functioning interactions may account for disappointing and inconsistent results when probiotics have been used to treat vulvovaginal candidiasis and antibiotic-associated diarrheas: merely adding organisms to a complex system—even in large amounts—can be insufficient to lead to a healthy community structure [14, 15].

5. INTEGRATING THE CONCEPTUALIZATIONS: FUTURE RESEARCH ON THE HUMAN MICROBIOTA AND HEALTH

The Human Microbiome Project (HMP) is a major roadmap initiative of the National Institutes of Health (NIH) [8]. Each NIH institute has been exploring various ways to meet the goals of the initiative, primarily from an organ viewpoint, in keeping with the organization of the institutes by disease or organ system. As the HMP moves forward, it would benefit from the development of an overall conceptual framework for structuring the research agenda, analyzing the resulting data, and applying the results in order to improve human health. Given the complexity of interactions among organisms in the human microbiota and the complexities and variations of human hosts and the organisms that inhabit those hosts, a catalog of microbes even from a range of multiple, diverse, individuals is only a first step towards the ultimate goal of manipulating human microbiota to prevent and treat disease. Further progress will require understanding the *drivers* of change in human microbiota that lead to disease states, particularly the underlying mechanisms and functions of microbiota, and how to establish and maintain communities consistent with health. Understanding the mechanisms and functions that process inputs and lead to outputs will enhance our ability to consistently manipulate the microbiota in the form of medical interventions and to minimize the unintended consequences of those interventions.

The level of complexity required to take a dynamic ecological view of human microbiota is daunting and will require collaborations among many disciplines including molecular biology, ecology, medicine, epidemiology, and mathematics. To fully understand the mechanisms that drive community structure and function, microbiota must be examined over time to determine the dynamics of its processes and over space to determine the interconnectedness of microbiota within an individual host and the range of microbiota among individuals. A comparison of microbiota among individuals living in countries with poorer sanitation to those with high levels of sanitation might be particularly interesting, in that normal, healthy, microbiota from less developed areas may regularly include helminthes. Moreover, these studies will require testing large numbers of diverse individuals, as the range of what is "healthy" or "normal" is probably very wide and may depend, in part, on the genetic make-up of the host and the associated environment. In addition, experimental approaches will

be essential to interpret descriptive studies. Experiments in well-controlled model systems such as bioreactors or animal models will be useful to isolate subsets of the interacting components depicted in the dynamic ecological community model (Figure 1(d)). Such experiments will provide a critical bridge between descriptions of highly diverse communities that change over time and space on one hand and the logistically intractable task of experimental investigation of all possible interaction pathways in such communities. Isolating key components of communities for intensive study of interactions has been very successful in understanding the ecology of macrocommunities [16–19]. Finally, mathematical models that require specification of the hypothesized underlying systems will enable conduct of simulation experiments to understand direct and indirect effects. The validity of simulation experiments depends heavily on the data available to "dock" the model. All of these approaches should lean heavily on well-developed ecological and evolutionary theories to form hypotheses and testable, quantifiable predictions.

Neither of the two extreme conceptualizations of the human microbiota, the multicelled organ and the ecological community model, are likely to be the most useful; integrated conceptualizations may be most appropriate for different research questions or clinical problems. Regardless of our conceptualization, however, we need to recognize that implicit assumptions yield different predictions on the impact of microbiota function on human health and move the research agenda in different ways. As the biomedical community moves into this rapidly burgeoning area, funds should be set aside to explore and develop theoretical underpinnings that draw on existing ecological and evolutionary theories and, thus, hasten efforts towards the ultimate goal of maintaining a healthy microbiota to maintain human health.

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Review Article

Application of Ecological Network Theory to the Human Microbiome

James A. Foster,[1,2] Stephen M. Krone,[2,3] and Larry J. Forney[1,2]

[1] *Department of Biological Sciences, University of Idaho, Moscow, ID 83844-3051, USA*
[2] *The Initiative for Bioinformatics and Evolutionary STudies (IBEST), University of Idaho, Moscow, ID 83844-3051, USA*
[3] *Department of Mathematics, University of Idaho, Moscow, ID 83844-3051, USA*

Correspondence should be addressed to James A. Foster, foster@uidaho.edu

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In healthy humans, many microbial consortia constitute rich ecosystems with dozens to hundreds of species, finely tuned to functions relevant to human health. Medical interventions, lifestyle changes, and the normal rhythms of life sometimes upset the balance in microbial ecosystems, facilitating pathogen invasions or causing other clinically relevant problems. Some diseases, such as bacterial vaginosis, have exactly this sort of community etiology. Mathematical network theory is ideal for studying the ecological networks of interacting species that comprise the human microbiome. Theoretical networks require little consortia specific data to provide insight into both normal and disturbed microbial community functions, but it is easy to incorporate additional empirical data as it becomes available. We argue that understanding some diseases, such as bacterial vaginosis, requires a shift of focus from individual bacteria to (mathematical) networks of interacting populations, and that known emergent properties of these networks will provide insights that would be otherwise elusive.

1. INTRODUCTION

The microbiota normally associated with the human body have an important influence on human development, physiology, immunity, and nutrition [1–6]. Also, communities of commensal and mutualistic bacteria associated with the human body constitute the first line of defense against infection by competitively excluding invasive nonindigenous organisms that cause disease. Yet despite their importance, surprisingly little is known about the composition of resident communities, how they differ between individual hosts or host environments, or such ecological relationships of constituent members as trophic interdependencies. Even so, human associated communities are likely to resemble those found in other habitats in at least four fundamentally important ways. First, natural microbial communities tend to be diverse in terms of species composition and physiological potential. Second, the flow of energy and nutrients through the system follows basic principles of microbial physiology, which results in the existence of trophic webs. Third, nutritional interdependencies exist wherein the "cross-feeding" of various vitamins, amino acids, and other cofactors occurs. And fourth, all ecological niches are occupied resulting in a relatively stable community composition. Armed with this information one can begin to postulate how external forces (e.g., invasive species such as nonindigenous microorganisms and pathogens) or treatments (e.g., the administration of antibiotics or changes in host diet) might affect the species composition and function of microbial communities that constitute the human microbiome.

Microbial communities can be viewed as mathematical networks with structural features that reflect how the networks developed and predict their responses to perturbations. In this paper, we will introduce the basic mathematical foundations of networks and briefly summarize some of their important structural properties. This approach to understanding microbial communities of the human microbiome is admittedly speculative, largely because of the lack of knowledge about community composition and species interactions in the human microbiome. Even so, it

is based on a growing body of research on evolving networks and may constitute a useful conceptual framework for understanding how these communities help maintain human health and how disturbances of the community structure and function could increase susceptibility to infectious disease. To illustrate the importance of ecological networks in the human microbiome, we will describe the biology of microbiota of the human vagina and how disturbances to these communities may account for the clinical syndrome known as bacterial vaginosis.

2. MUTUALISTIC RELATIONSHIPS OF THE VAGINAL MICROBIOME

The human vagina and the bacterial communities that reside therein, form a finely balanced mutualistic association. Previous studies indicate that indigenous bacterial populations play a key role in preventing colonization by "undesirable" organisms, including those responsible for bacterial vaginosis, yeast infections, sexually transmitted diseases, and urinary tract infections [7–12]. Historically, lactobacilli have been thought to be the keystone species of vaginal communities in reproductive-age women, both in the sense of being the dominant species and in the sense of being the species with the greatest impact on the vaginal ecosystem. These microorganisms benefit the host by producing lactic acid as a fermentation product that accumulates in the environment and lowers the pH to ~4.5 [13]. While a wide range of other species are known to be members of vaginal bacterial communities, their ecological functions are largely unknown, as is the total number of species present. The host provides benefit to the microbial communities by providing all the nutrients needed to support bacterial growth. This is of obvious importance since bacteria are continually shed from the body in vaginal secretions, and bacterial growth must occur to replenish their numbers. Some of the required nutrients are derived from sloughed cells, while others are from glandular secretions. Surprisingly, the precise composition and the concentrations of various constituents are poorly understood, and this is an important knowledge gap. Nonetheless, the data available indicate that there are proteins and carbohydrates of various kinds in vaginal secretions, as well as urea, K+, Na+, and, Cl− [14] and it seems likely that various amino acids, peptides, and monosaccharides are also present. The symbiotic relationships between host and bacterial populations seem likely to be mutualisms, with each species benefiting from the presence of the other. (It should be noted that bacterial populations of the human microbiome are often referred to as commensal bacteria, which implies that only one member of the association benefits while the other is unaffected. In many cases, if not all, this is probably an incorrect characterization of the ecological relationship between the two members.)

3. ETIOLOGY OF BACTERIAL VAGINOSIS: A DISEASE LINKED TO COMMUNITY DISTURBANCES

Bacterial vaginosis (BV) is a syndrome that is often characterized as a disturbed microbial community [15] although it is most often diagnosed based on the occurrence of three of the following four criteria: (a) homogeneous, white adherent vaginal discharge; (b) a vaginal pH > 4.5; (c) detection of "clue cells" by microscopy; and (d) the presence of an amine odor upon addition of KOH to vaginal secretions [16]. Intensive efforts to identify etiological agents have thus far been unsuccessful, and it has been suggested that the disturbed communities themselves may account for the observed symptoms.

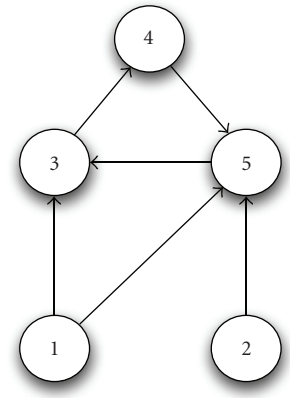


Figure 1: A hypothetical trophic web with five species. Species 1 and 2 are "grazers" at the bottom level, which acquire nutrients directly from the environment and provide nutrients to species 3 and 5. Species 3, 4, and 5 form a dependent cycle, with 3 and 5 at the second level of the web and 4 at the final level.

BV has important consequences for women's health. The prevalence of BV among reproductive-age women ranges from 29% in U.S. population-based surveys to over 50% in rural Ugandan villages [17]. It has been associated with an increased risk of preterm delivery, first trimester miscarriage in women undergoing in vitro fertilization, chorioamnionitis, endometritis, and pelvic inflammatory disease (PID) [18–23]. Moreover, BV increases the risk of acquiring *Neisseria gonorrhoeae* and other sexually transmitted diseases [11, 24] including HIV [8, 25].

Historically, BV has been associated with depleted numbers of *Lactobacillus* spp. and an elevated vaginal pH [26, 27]. However, this simple view has been challenged [28] by recent findings that showed that the vaginal communities of many normal and healthy Caucasian, black, and Japanese women lack appreciable numbers of *Lactobacillus* spp., but instead include other taxa of lactic acid producing bacteria (LAB) [29, 30]. This has two important implications. First, an important ecological benefit to the host—maintenance of a low vaginal pH—is conserved among individual women, although the species composition of the microbial communities can vary. This is consistent with the consensus viewpoint that a low pH environment in the vagina is a key mechanism for defending the host against potential pathogens. And second, factors that alter the species composition, the physiological activities of bacterial populations, or the overall community function (reducing

the pH of the local environment), could lead to the symptoms associated with BV.

Previous studies have established that several distinct kinds of vaginal communities occur in Caucasian and black women in North America [29, 30], and Japanese women in Tokyo, Japan [Zhou, 2008; unpublished]. Since vaginal bacterial communities differ in species composition [30–33], they are likely to differ in how they respond to disturbances, and disruptions of ecological equilibria may increase risk to invasion by infectious agents. Conceptually this is important since vaginal communities continually experience various kinds of chronic and acute disturbances such as the use of antibiotics and hormonal contraceptives, sexual intercourse, douching, menstruation, and many others.

4. NETWORK APPROACHES TO UNDERSTANDING THE HUMAN VAGINAL MICROBIOME

By analogy with microbial communities in other ecosystems, we postulate that a complex food web exists among member species of vaginal bacterial communities, and that various populations occur in distinct trophic levels. Given that the resource pool is diverse (as described above), it is reasonable to project that the species composition, expressed physiological traits, and kinds of nutritional interdependencies of vaginal bacterial populations are strongly influenced by the kinds of nutrients available in the vagina. This implies that host characteristics could be an important "driver" of microbial ecosystems, while the members of the microbial community are stratified in such a way that one or more populations are primary consumers, while others consume their metabolites, and so on. The result is a "network" that reflects the flow of energy and nutrients through the ecosystem in which the configuration and strengths of ecological interactions determine the stability and resilience of the community. Such networks are commonly referred to as microbial trophic webs (Figure 1).

In dissimilatory microbial trophic webs a few species specialize in breaking down larger, more complex organic molecules into smaller molecules [34, page 102]. These specialists may require little assistance from other species. There are likely to be more pathways (and microbial species) able to metabolize these smaller molecules, and still other species to consume the resulting metabolites. If complete mineralization of carbon sources occurs, then carbon dioxide is produced, but in the absence of suitable terminal electron acceptors, fermentation products (such as lactic acid) accumulate in the environment.

Some populations in dissimilatory consortia may have secondary roles that regulate the growth and function of other populations in the consortia. For example, one population may produce growth factors such as amino acids, peptides, or vitamins that are used, and sometimes required, for other populations to grow. Indeed, lactobacilli are notoriously fastidious and have complex nutritional requirements [35–37]. This sort of nutritional cross-feeding represents a "positive feedback loop." In contrast, various small molecules that disrupt membrane function, antibiotics, and bacteriocidal proteins [38] constitute "negative feedback loops." These positive and negative feedback loops play a role in governing the size of different bacterial populations and their activities. To understand such a complex network, one may very well have to adopt a systems approach such as that described below [39].

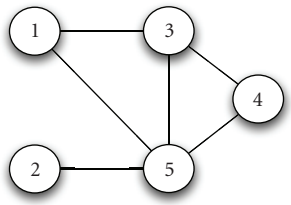
Since there may be very few specialist species at the base of microbial trophic webs, assembly rules may be strongly influenced by priority effects. A priority effect [40, page 247] is the influence that one species exerts on whether another can endure in an environment, simply by being there first. Assembly rules describe the order in which species tend to occupy habitats. For example, the first species to colonize a microbial ecosystem that specializes in catabolizing the dominant nutrient or nutrients may determine which new nutrients are then available, and thereby constrain which other species can successfully colonize the habitat and persist.

5. MATHEMATICAL REPRESENTATION OF NETWORKS

Microbial trophic webs of the human microbiome are instances of a more general abstract structure: mathematical networks. In ecology, trophic webs are typically visualized as nodes on a graph representing individual species that are connected by directed edges that indicate who is dependent on whom for nutrition. These webs are sometimes called "food webs," with a tacit assumption that the relationship is one of who eats whom. Predatory-prey relationships exist at all scales of life. But both macro- and microbial trophic relationships are much richer than predation alone. For example, species interactions often involve cross-feeding, where each species acquires nutrients, or compounds that inhibit growth, that are produced by other species. In microbial systems, these indirect products are molecular, while in macrobial systems they may be much larger.

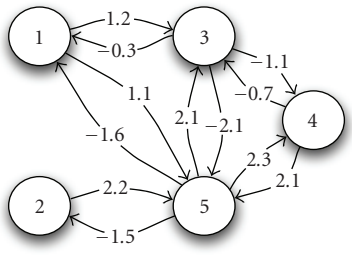
Collections of nodes and edges such as those used to visualize trophic webs are instances of mathematical networks. One useful characteristic of this mathematical abstraction is its general applicability. Any collection of "individuals" and "relationships" can be expressed and analyzed as a network, regardless of details about the individuals or the relations. In particular, networks are not limited to trophic webs.

The simplest mathematical networks indicate only whether or not two nodes are connected by an edge by setting the corresponding "adjacency" term to 1 or 0; thus, $a_{i,j}$ is set to 1 if the ith individual is connected to the jth individual, and to 0 if they are not connected. These networks are often summarized in an adjacency matrix A with the term $a_{i,j}$ appearing in the ith row and jth column. These connections are undirected when the matrix is symmetric, meaning that $a_{i,j} = a_{j,i}$ (visually, reflecting the matrix across the main diagonal leaves it unchanged). One can represent additional information about the relation between two individuals by letting the matrix entries be numbers other than 0 and 1 (Figure 2). For example, an ecological network could correspond to a system of Lotka-Volterra differential equations describing species interactions $du_i/dt = u_i(r_i + \sum_j a_{i,j}u_j)$ where r_i is the intrinsic growth rate of species i and $a_{i,j}$ is the "effect" of species j on species i.



	1	2	3	4	5
1	0	0	1	0	1
2	0	0	0	0	1
3	1	0	0	1	1
4	0	0	1	0	1
5	1	1	1	1	0

Figure 2: Mathematical network with undirected edges, representing the structure of the trophic web in Figure 1, pictorially and as an equivalent adjacency matrix. The connectivity of the nodes is one for node 2, two for nodes 1 and 4, three for node 3, and four for node 5 (which is a hub node).



	1	2	3	4	5
1	0	0	−0.3	0	−1.6
2	0	0	0	0	−1.5
3	1.2	0	0	−0.7	2.1
4	0	0	−1.1	0	2.3
5	1.1	2.2	−2.1	2.1	0

Figure 3: Directed graph representing (hypothetical) strengths of species interactions and the corresponding matrix of interaction strengths. Positive (negative) values indicate increase (decrease) in receiving species' fitness. Units of interaction are unspecified in this example, but may be observed changes in biomass. For example, species 1 may produce a metabolite beneficial to species 3($a_{31} = 1.2$), while 3 occasionally harms 1($a_{13} = -0.3$) while consuming the metabolite. Species 3 and 4 are competitors, 5 and 4 are mutualists, and other pairs resemble predator/prey.

Here, the interactions are described by a matrix A = $(a_{i,j})$ of real numbers. For example, if i is a prey species and j a predator, we would have $a_{i,j} < 0$ and $a_{j,i} > 0$ (Figure 3). Food webs are special cases of ecological networks in which the interactions are all of predator-prey type with predators in one trophic level feeding on prey from a lower level (Figure 1).

It can extremely difficult to obtain information about trophic interactions (especially interaction strengths) in real ecological networks. However, it is becoming easier to gather quantitative data for networks given advances in high throughput sequencing technologies and sophisticated computational biology algorithms. For example, as more annotated genomes become available, it becomes easier to form hypotheses about potential metabolic pathways. It is encouraging that genome annotation, comparative genomics, and hypothetical pathway reconstruction are autocatalytic, each improving the accuracy and efficiency of the others. With such positive feedback, we anticipate that it will become increasingly easy to parameterize network models accurately.

Surprisingly, however, one does not need accurate parameters in an abstract network, since the network structure alone can tell one a great deal about the system that it represents. A characteristic that matters in all networks is the number of links or "connectedness" of each node, and this of course varies from one node to another within a network [41]. For example, a property of many natural networks is that they are "scale free," roughly meaning that there is no single degree of connectedness that is characteristic of the network. In scale free networks, most nodes are connected to a small number of other nodes, and a small number of nodes act as "hubs" in that they are connected to many nodes. A scale free network is usually robust to the removal of randomly selected nodes but can be violently destabilized when hub nodes are removed. In a very real way, these hubs are analogous to keystone species in biological ecosystems. When the population size or activity of a keystone species is changed, or the species is entirely removed, dramatic changes occur in the varieties and population densities of all other species in the community.

It is even possible to learn a great deal with neither accurate graph topologies nor extensive empirical parameterization. Theoreticians construct artificial networks with different types of assembly rules, essentially reverse engineering the abstractions of natural networks. This discipline has been aptly termed the statistical mechanics of complex networks [42].

Remarkably, two informative properties consistently emerge from such simulations. First, in both real and simulated ecological networks one finds a "many weak, few strong" pattern in which most, but not all, species interactions are weak. Specifically, the average interaction strength (average of $|a_{i,j}|$'s) times the square root of the average number of edges per node, often converges to a constant over time [43–45]. A second "emergent" property is that networks tend to evolve to the point where they are at the brink of instability, being in some sense most productive when living on the edge. Extinction events in an ecological network, either by "natural" means or by artificially removing nodes, typically lead to occasional avalanches of secondary extinctions [43, 46]. In fact, this is where the "many weak, few strong" pattern comes from: extinctions of most species have minor effects, while removal of those species that are strongly connected can destabilize the entire ecosystem, resulting in a cascade of extinctions. This instability essentially arises from "successful" interactions that form in the evolving network through, for example, collaborative consortia. Such interdependencies in collaborations can ultimately lead to instability, since disturbing any one species in the consortium can affect many others.

These features are among the self-organizing principles that reveal themselves in many natural and simulated networks. This suggests that the study of evolving networks can enable one to predict microbial ecosystem behavior, even without quantifying all the details of the interactions between species in a complex ecological network. When studying the complex communities of the human microbiome, where very little is known, this is a great advantage.

The application of theoretical network modeling to real ecological networks has thus far been focused primarily on attempts to capture observed features of the networks. One of the reasons for the rapid growth of network theory is the stunning regularity with which certain course-grained

"topological" properties emerge in real ecological (and social and technological) networks. These properties, depending on global characteristics of the network such as the number of links, connectance, and so on, appear in such a wide variety of settings that it was natural to try to come up with simple models that would produce the same features. Thus, there appear both static and dynamic models that reproduce some of the topological properties of real networks [44, 45, 47, 48]. As one moves to more fine-grained properties (e.g., degree distribution) or seeks to develop predictive models, however, one must rely increasingly on dynamic models that carry more details about the system. Most studies of real ecological networks are restricted to food webs wherein all links between species are of the predator-prey type.

An example of how the models are applied to the real networks is in trying to understand the stability of an ecosystem to extinctions or other perturbations. Some models predict stability or instability based on the connectivity of the network. For example, the scale free property observed in many real food webs carries with it a prediction of stability under removal/extinction of weakly connected species but become highly unstable with avalanches of secondary extinctions when one of the few highly connected species is removed. There are limitations, however, to our current understanding since the stability analyses have been rather restricted and the models lack some details that could play essential roles.

6. SUMMARY

We have argued that mathematical networks provide a system-level approach to characterizing microbes and microbial interactions, which may improve descriptions of how consortia in the human microbiome are related to disease etiology, diagnosis, and treatment. Networks may capture specific biological information, such as how nutrients flow through the species in a microbial consortium. Ecological principles applied to such microbiome-specific networks are likely to constrain how the microbiome will respond to invasive species or to purportedly benign disturbances such as antibiotic treatment. Moreover, network structure sometimes suffices to indicate how a consortium is likely to have evolved or to identify keystone species, even when interaction strengths have not been quantified. This is particularly useful when detailed data on the constituents and species interactions in a consortium are unavailable. In short, for some human diseases such as bacterial vaginosis, it may be more useful to examine the forest, rather than the trees.

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Review Article

Interactions of the Intestinal Epithelium with the Pathogen and the Indigenous Microbiota: A Three-Way Crosstalk

C. V. Srikanth[1,2] and Beth A. McCormick[1,2]

[1] *Department of Pediatric Gastroenterology and Nutrition, Mucosal Immunology Laboratories, Massachusetts General Hospital, Charlestown, MA 02129, USA*
[2] *Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, USA*

Correspondence should be addressed to Beth A. McCormick, mccormic@helix.mgh.harvard.edu

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The mucosal surfaces of the gastrointestinal tract harbor a vast number of commensal microbiota that have coevolved with the host, and in addition display one of the most complex relationships with the host. This relationship affects several important aspects of the biology of the host including the synthesis of nutrients, protection against infection, and the development of the immune system. On the other hand, despite the existence of several lines of mucosal defense mechanisms, pathogenic organisms such as Shigella and Salmonella have evolved sophisticated virulence strategies for breaching these barriers. The constant challenge from these pathogens and the attempts by the host to counter them set up a dynamic equilibrium of cellular and molecular crosstalk. Even slight perturbations in this equilibrium may be detrimental to the host leading to severe bacterial infection or even autoimmune diseases like inflammatory bowel disease. Several experimental model systems, including germ-free mice and antibiotic-treated mice, have been used by various researchers to study this complex relationship. Although it is only the beginning, it promises to be an exciting era in the study of these host-microbe relationships.

1. INTRODUCTION

A mature human gut harbors a vast number of bacterial residents referred to as the commensal microflora or more recently as "microbiota." It has been estimated that this microbiota is made up of more than 10^{14} individual bacteria comprising over 500 different species [1]. Notably, the composition of the microbiota is individual specific and the type of species residing in the gastrointestinal (GI) tract varies with the host organism's age, diet, and health status [2]. In fact, the total number of microbes in the human GI tract far exceeds (>10–100 times) the sum of all our somatic and germ cells. The biological outcome of this vast and complex population of microbes is that their genes (termed the microbiome) synthesize about 100 times more proteins than the somatic cells of their host [3].

Not surprisingly, the human intestine is more densely populated with microorganisms than any other organ and is a site where they exert a strong influence on human biology. This is because the intestinal mucosa serves as the primary border between the immune system and the external environment, and in addition plays a central role in host-commensal flora interactions. Accumulating evidence indicates that the gut microbiota is instrumental in supporting energy metabolism and immune function of the host. More recent studies suggest that the commensal microbiota play an important role in the development of numerous conditions, including obesity [4, 5], diabetes [6], nonalcoholic fatty liver disease [7], inflammatory bowel disease [8], and perhaps cancer [9]. Unfortunately, the immense complexity of gut flora together with its highly complicated interactions with intestinal epithelium makes it a recalcitrant system to study. Although largely unexplored, our gut microbiota plays an intricate and underappreciated pivotal role for our health and well-being. In this review we will discuss new developments in the field that highlight the cellular and molecular basis of the crosstalk between the host, the commensal microbiota, and pathogenic bacteria in a healthy as well as a diseased GI tract.

2. ROLE OF THE MICROBIOTA IN THE GASTROINTESTINAL TRACT

The microflora of the intestinal microenvironment as a unit provides important protective, metabolic, and trophic functions. Resident bacteria serve a central line of resistance to colonization by exogenous microbes, and thus assist in preventing the potential invasion of the intestinal mucosa by an incoming pathogen. This protective function is known as the barrier effect or colonization resistance and serves a number of important roles. For instance, adherent nonpathogenic bacteria can often prevent attachment and subsequent entry of suspected pathogens into epithelial cells, as well as compete for nutrient availability. The commensal microbiota also helps maintain GI nutrient homeostasis by administering and consuming all resources. For example, dietary nutrients are absorbed by the gut and together with various nonnutrient compounds produced by the microbiota are cometabolize by host enzymes, such as cytochrome P450 and conjugating enzymes in the liver [10]. The resulting metabolites that are derived from both host and microbial processes are returned to the gut by the bile for further metabolism or excretion [11]. This mutual and beneficial relationship helps to dampen unwanted overproduction of nutrients, which could potentially support intrusion of microbial competitors with a potential pathogenic outcome for the host [12].

Quite remarkably, an absence of intestinal bacteria is associated with reduction in mucosal cell turnover, vascularity, muscle wall thickness, motility, baseline cytokine production, digestive enzyme activity, and defective cell-mediated immunity [13]. Indeed, comparative studies in germ-free and conventional animals have established that the intestinal microflora is essential for the development and function of the mucosal immune system during early life, a process that is now known to be important to overall immunity in adults. For example, it has been well established that the number of intraepithelial and lamina propria T cells is lower in germ-free animals, a feature that is reversed upon the restoration of the normal flora [14]. Likewise, levels of secretory IgA are low in the intestine of germ-free animals but are markedly increased upon intestinal colonization of the commensal bacterium, *Bacteroides thetaiotamicron* [15]. Furthermore, the intimate relationship between the commensal microbiota and the intestinal epithelium are involved in shaping the memory mechanisms of systemic immunity, such as oral tolerance. This was initially recognized by the discovery that the systemic response to a specific pathogen can be abrogated after ingesting the antigen; this effect continues for several months in conventionally colonized mice, whereas in germ-free mice systemic unresponsiveness persists for only a few days [16]. Therefore, the innate immune system discriminates between potential pathogens from the commensal microbiota by inducing tolerance to microbial epitopes. This, in turn, dampens responses to commonly encountered foodstuffs and other environmental antigens. Collectively, these examples help to illustrate the important concept that the commensal microbiota profoundly influence the development of the gut mucosal immune system and are essential in preventing exogenous pathogen intrusion.

The intestinal microflora also makes important metabolic contributions by producing vitamin K, folate, and short-chain fatty acids (a major energy source for enterocytes), and mediates the breakdown of dietary carcinogens as well [2, 17]. Perhaps the major metabolic function of the colonic microflora is the fermentation of nondigestible carbohydrates. These nondigestible carbohydrates include large polysaccharides (i.e., resistant starches, pectins, cellulose), some oligosaccharides that escape digestion, as well as unabsorbed sugars and alcohols. The primary metabolic endpoint of such fermentation is the generation of short-chain fatty acids (acetate, proprionate, butyrate). A fundamental role of short-chain fatty acids on colonic physiology is their trophic effect on the intestinal epithelium. Therefore, short-chain fatty acids appear to play an essential role in the control of epithelial cell proliferation and differentiation in the colon. Recent studies have also shown effects of butyrate on intestinal barrier function [18]. Moreover, it has been shown that commensal bacterial can modulate gene expression in the host in order to create a sustainable environment for themselves, while at the same time prevent the growth of other competitive bacteria within the intestinal ecosystem [15].

For the host to thrive and produce more gut residents, the gut microbial ecosystem must be functionally stable over time despite the internal dynamics of the community. Constituent bacteria are expected to have a high degree of functional redundancy between species, so that the loss of one lineage does not adversely impact the homeostatic balance of the intestinal microenvironment [19]. While it is unclear how the selective pressures, microbial community dynamics, and the intestinal microenvironment shape the genome and subsequent functions of members of the gut microbiota, there are some exciting new developments in the field. For example, Gordon et al. have introduced the provocative concept that the evolution of the gut microbiome also likely plays a significant role in shaping the evolution of humans [19]. This tenet is founded on experiments in which this team of investigators sequenced the genomes of two gut-dwelling Bacteroidetes and compared their genomes to the genomes of other bacteria that live both inside and outside of the human body. Quite remarkably, they discovered that lateral gene transfer, mobile genetic elements, and gene amplification play an important role in affecting the ability of the Bacteroidetes to vary their cell surface, sense their environment, and harvest nutrient resources present in the distal intestine [19]. Importantly, these findings lay the conceptual groundwork to suggest that adaptation to the gut ecosystem is a dynamic process that includes acquisition of genes from other microorganisms, and further underscores the significance of considering the evolution humans from the perspective of the evolution of the microbiome [19, 20].

3. RESTRICTING PATHOGENS AND COMMENSAL FROM INVADING BEYOND THE MUCOSAL SURFACE

The host is protected from potentially harmful enteric microorganisms by the physical and chemical barriers created by the intestinal epithelium that are primarily comprised of

absorptive villus enterocytes [21]. The apical surface of the enterocytes are highly differentiated structures consisting of rigid, closely packed microvilli whose membranes contain stalked glycoprotein enzymes [22, 23]. In addition, the tips of enterocyte microvilli are coated with a 400–500 nm thick meshwork referred to as the filamentous brush border glycocalyx [24] and is composed of highly glycosylated transmembrane mucins [25, 26]. The intestinal epithelial barrier is also composed of enteroendocrine cells, goblet cells, and Paneth cells. Microfold (M) cells are also present in the follicle-associated epithelia where they represent a morphologically distinct epithelial cell type whose primary function is in the transport of macromolecules, particles, and microorganisms from the lumen to underlying lymphoid tissue [27, 28]. Intercellular junctional complexes that are composed of tight junctions, adherens junctions, and desomosomes maintain the integrity of the epithelial barrier. The most apical components of the junctional complex are the epithelial tight junctions, which are highly regulated and serve to create a semipermeable diffusion barrier between individual cells (Figure 1(a)). Collectively, these features facilitate the intestinal epithelium to act as a physical barrier to prevent unwanted bacteria from gaining access to the host.

The intestinal epithelium also provides a unique surface that is armed with a bounty of specialized cells that produce mucus, antimicrobial peptides, and antimicrobial molecules, which together form the front line of defense against pathogenic microorganisms (Figure 1(a)). The mucus layer is secreted by the goblet cells and this layer overlies the intestinal epithelium to create a physical blockade against offending enteric microbial pathogens. For example, it has been demonstrated that secreted mucus acts as a barrier to *Yersinia enterocolitica* [29], rhesus rotavirus [30], and *Shigella flexneri* [31]. The commensal microbiota has also been found to regulate the production of intestinal mucins, which consequently inhibits the adherence of numerous pathogenic bacteria to intestinal epithelial cells [32–34]. Paneth cells are another important cell type that are involved in intestinal defense against potential harmful pathogenic bacteria. These cells are present at the base of the crypt of Lieberkühn [35] and have been shown to produce a number of antimicrobial peptides. In addition, the gastrointestinal expression of antimicrobial peptides is evolutionarily conserved [36], and to date, α-defensins (HD), β-defensins (hBD), and cathelicidins have been identified in humans [37]. Paneth cells also produce a number of antimicrobial molecules, including lysozyme, phospholipase A_2, and angiogenin-4 (reviewed in [37]). Therefore, it is inferred by numerous studies that Paneth cells are able to control the bacterial ecosystem (Table 1).

Angiogenin-4 is expressed mainly in the small intestine, cecum, and colon and acts on Gram-positive bacteria [49, 50]. However, most antimicrobial peptides expressed by mammalian epithelial cells are members of peptide families that mediate nonoxidative microbial cell killing by phagocytes [50]. These amphipathic molecules interact with and lyse bacterial membranes [55]. Defensins generally possess a broad range of antimicrobial activity (Table 1). In particular, human intestinal defensin-5 has been shown to kill *Listeria monocytogenes*, *E. coli,* and *Candida albicans* [40]. Additional evidence supporting a critical role for defensins in vivo was demonstrated in a study utilizing human defensin-5 transgenic mice; these mice exhibited marked resistance to oral challenge with virulent *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) [39]. The intestinal epithelial cells also express another class of antimicrobial peptide, the cathelicidins (LL-37/Cap18), in which a cathelin domain is linked to a peptide with antimicrobial activity [56]. LL-37 is expressed within the epithelial cells located at the surface and upper crypts of normal human colon. Although little or no expression is seen within the deeper colonic crypts or within epithelial cells of the small intestine, studies in mice have determined these molecules to be protective against bacterial pathogens [47]. Interestingly, the expression of these factors, unlike the angiogenins, is not induced by the presence of pathogenic bacteria but rather their secretion is triggered by the commensal microbiota and/or their derivatives. A recent addition to this growing list of intestinal antimicrobial includes RegIIIγ, which has been shown to be toxic to Gram-positive bacteria [52]. RegIIIγ is a C-type lectin that binds to the carbohydrate moiety of bacterial cell wall constituent, petidoglycan. Recent studies have further shown that the expression of RegIIIγ is strongly dependent upon the presence of the gut microflora since in germ-free mice RegIIIγ expression is severely repressed [53] (Table 1).

The intestinal epithelium also provides a surface where the host can sense the microbial microenvironment in order to elicit an appropriate defense response by releasing an array of signaling molecules (i.e., chemokines and cytokines). These molecules then trigger the recruitment of leukocytes to initiate an early inflammatory response. Paradoxically, however, although continuously exposed to Gram-positive and Gram-negative bacteria and their products (i.e., lipopolysaccarhide (LPS), peptidoglycan, and lipoprotein) the normal healthy intestinal mucosa maintains a mechanism of hyporesponsiveness to the lumenal microbiota and their products. Exaggerated inflammatory responses in the absence of pathogenic bacteria would be otherwise deleterious [57, 58]. Accordingly, the normal intestinal epithelial host defenses are able to accurately interpret the complex microbial environment in order to discriminate between permanently established commensal microbes and episodic pathogens.

At the core of this strategy the endogenous microbiota all share "self" signature molecules termed microbe-associated molecular patterns [59]. However, upon infection of a pathogenic organism, the host immune response is activated by the specific recognition of "nonself" molecular structures known as pathogen-associated molecular patterns. The epithelial cells are able to sense the microenvironment within the gut by means of pattern recognition receptors (PRRs) that include Toll-like receptors (TLRs) and nucleotide-binding oligimerization domain (NOD) proteins [38, 60–63]. TLRs are evolutionary conserved and are characterized by an extracellular leucine rich repeat (LRR) domain (involved in ligand recognition), as well as an intracellular

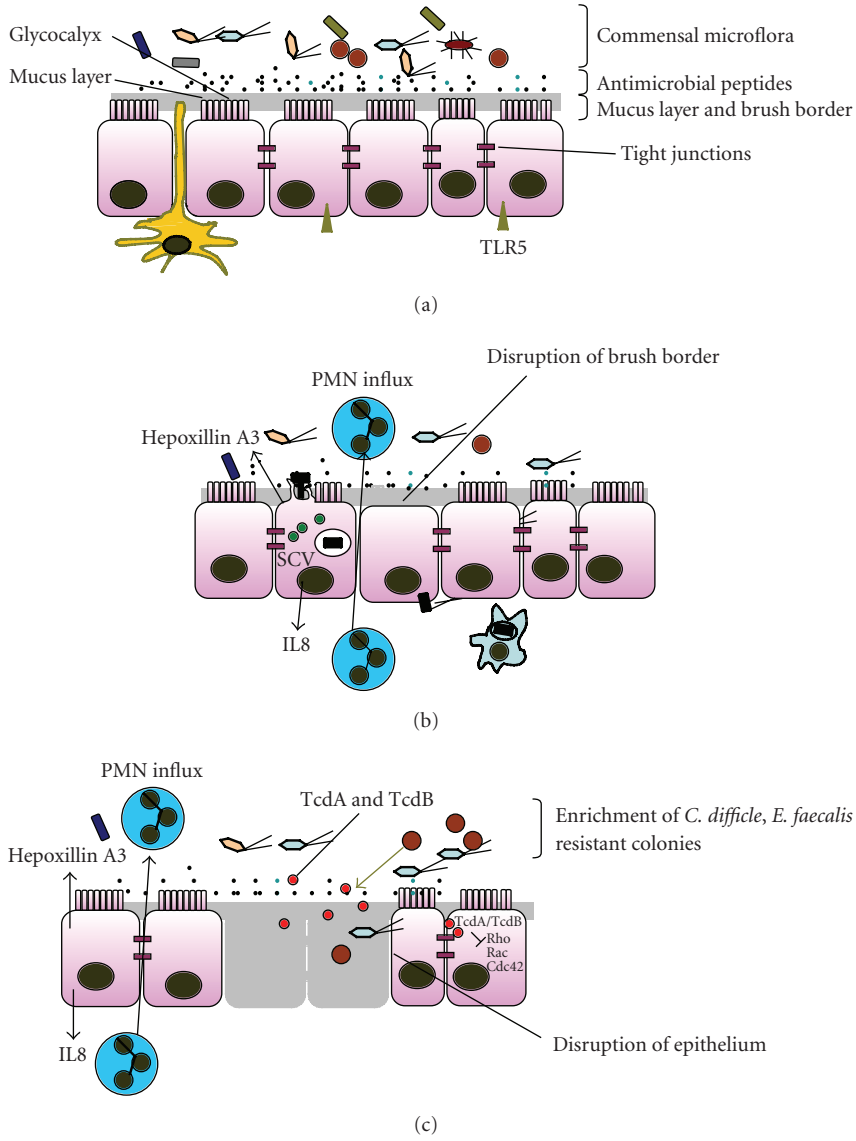

Figure 1: (a) Healthy epithelial surface. A healthy intestinal epithelial surface acts as a physical and biochemical barrier with key features including the apical brush border, the mucus layer, the presence of antimicrobial peptides (blue black dots) in the lumen, the glycocalyx, and the epithelial tight junctions. Also seen in the illustration are numerous commensal bacteria and a dendritic cell sampling the lumen with its extended dendrites (yellow). (b) Key features of *S. typhimurium* infected epithelium. Such host pathogen interactions involve translocation of bacterial effectors (green circles) into the epithelial cells, membrane ruffling, bacterial endocytosis, and SCV formation. Chemoatractants are secreted by the epithelial surface that leads to PMN influx. SCV: Salmonella containing vacuole. (c) Intestinal epithelial surface of an antibiotic-treated patient showing enrichment of a set of antibiotic resistant members of the commensal microflora (light blue and brown) such as *C. difficle* and *E. faecalis*. The *C. difficle* proteins, TcdA and Tcdb (red circles) act intracellularly as glycosyltransferases and inhibit Rho, Rac, and Cdc42. The effect of these modifications lead to actin condensation, transcriptional activation of several genes and apoptosis. Other mechanisms that are triggered include basolateral IL8 secretion, apical Hepoxillin A synthesis, and PMN influx in the apical surface.

Toll/interleukin-1 receptor-like domain (involved in proinflammatory signal transduction) [60, 64–66]. In addition, two NOD proteins (NOD1 and NOD2) function as intracellular sensors of bacterial products in the induction of an inflammatory response [60, 64–67].

These PRRs recognize bacterial factors, such as LPS, lipoproteins, flagellin, unmethylated-CpG DNA, and a large number of other specific components. Regulation of the expression and the specific location of TLRs and NODs in intestinal epithelial cells fosters efficient immune recognition of the commensal microflora and maintains a delicate balance; permitting a basal level of signaling events to proceed, while at the same time restraining innate immune responses. For instance in a healthy intestine, epithelial cells express very little or no TLR2, TLR4, and CD14, and as a result minimizes the recognition of commensal LPS [68, 69]. TLR5, which recognizes bacterial flagellin, has been reported to be expressed exclusively on the basolateral surfaces of the

epithelial cells. This TLR is ideally positioned to detect its ligand, translocated flagellin [70]. Moreover TLR3, TLR7, TLR8, and TLR9 are expressed in the intracellular endosomal compartments [71]. These intracellular PRRs would not ordinarily encounter luminal commensal bacteria or those attached to the apical surface of intestinal epithelial cells but are well positioned to recognize pathogenic bacteria that actively breach the epithelial barrier. As an additional measure, commensal bacteria have the ability to induce the expression of intestinal alkaline phosphatase, which not only dephosphorylates dietary lipids but also dephosphorylates the LPS of commensal flora resulting in reduced toxicity in mammals [72].

Nonpathogenic microorganisms may also be able to selectively attenuate the NF-kB pathway as mechanism of intestinal immune tolerance. Neish et al. initially reported that colonization of a human model intestinal epithelium with certain strains of nonpathogenic bacteria could dampen the host cell responses to subsequent proinflammatory challenges by blocking the proinflammatory/antiapoptic NF-κB pathway [73]. This effect is mediated by the inhibition of IκB-α ubiquitination, which prevents regulated IκB-α degradation, NF-κB nuclear translocation, and subsequent activation of proinflammatory/antiapoptic genes. IκB-α ubiquitination is catalyzed by E3-SCF$^{\beta\text{-TrCP}}$ ubiquitin ligase [74], which is regulated via covalent modification of the cullin-1 subunit by the ubiquitin-like protein NEDD8 [75, 76]. Recently, it was determined that the interaction of nonpathogenic bacteria with epithelial cells results in the rapid loss of neddylated Cul-1 and consequent repression of the NF-κB pathway [77]. Collectively, this set of observations underscores the ability of intestinal bacterial communities to influence eukaryotic processes, and perhaps more specifically demonstrates inflammatory tolerance of the mammalian intestinal epithelia.

4. HOW PATHOGENS OVERCOME THE EPITHELIAL BARRIER

As described above, the intestinal epithelium has evolved a rather formidable fortress to guard against microbial invasion. However, through a process of coevolution, potential harmful enteric microorganisms have evolved counter strategies to hijack the cellular molecules and signaling pathways of the host to become potentially pathogenic. As an initial step in the infection process, certain enteric pathogens target specific epithelial cell structures, including glycoproteins and glycolipids, which serve as receptors for bacterial attachment [78]; thus, enabling them to exploit the underlying signal transduction pathway. Other strategies utilized by invading enteric pathogens, such as *S. typhimurium* and *Shigella flexneri* have evolved a sophisticated strategythat directs the entry of the enteric pathogen into intestinal epithelial cells. This process requires the expression of a bacterial type III protein secretion system (TTSS), the function of which is to deliver a set of effector proteins into the host cell [79–81]. These effector proteins co-opt host cell signal transduction cascades as a clever means of subverting normal host cell processes by triggering a marked rearrangement of the host cytoskeleton. This entry mechanism termed bacterial mediated endocytosis drives bacterial entry and facilitates the pathogen to cross the epithelial barrier as well as to induce a proinflammatory response [79–81].

The latter step in this process can be achieved by direct cytotoxic injury, intracellular migration, disruption of the epithelial tight junctions, or indirectly by inducing neutrophil infiltration. Although several bacterial pathogens have been able to modulate epithelial tight junctions to their own advantage, the direct interaction of a bacterial virulence factor on component proteins of the tight junction has been proposed only in a few instances [82]. It is well documented that anumber of enteric pathogens perturb the intestinal epithelial barrier and impact TER or paracellular permeability, most often with an alteration in the arrangement of tight junctional component proteins by mechanisms that are unique for different pathogens [82]. For example, *Clostridium difficile* toxins A and B enhance epithelial cell permeability by disrupting actin microfilaments within the perijunctional ring [83], and enteropathogenic *Escherichia coli* disrupt the epithelial barrier by the phosphorylation of myosin light chains [84]. With respect to *S. typhimurium*, in vitro models of infection have revealed an alteration of epithelial permeability and loss of barrier function, which involves rapid changes in both tight junction permeability and transcellular conductance [85, 86]. Recent studies further indicate that the *Salmonella* effector protein SigD (also called SopB), which is encoded in *Salmonella* pathogenicity island-1 (SPI-1), is able to elicit a reduction in epithelial barrier function, perhaps via activation of PKC [87]. Also, the effector proteins SopB, SopE, SopE2, and SipA are necessary to disrupt the epithelial barrier and alter the distribution of at least some tight junction proteins [88, 89]. Such perturbations in the components of the tight junction lead to enhanced bacterial translocation and infiltration of neutrophils across the intestinal barrier. Therefore, the ability to regulate the molecular composition of the tight junctions facilitates the pathogenecity of *S. typhimurium* by fostering its uptake and distribution within the host (Figure 1(b)) [85].

S. flexneri has a distinct mode of pathogenesis that involves entry into colonic epithelial cells from the basolateral surface [90], thereby requiring its relocation from the lumenal to the underlying surface of the epithelium. This translocation event has historically been attributed to the uptake and transport by M cells [91]. However, it has since been established that *Shigellae* are also capable of altering components of the tight junctional complex, allowing the bacteria to traverse the paracellular space to reach the basolateral surface; an event that also decreases barrier function [92]. Once at the basolateral surface, *Shigellae* rapidly invade and disseminate through the epithelium, causing a further decrease in barrier function [92–94] through the action of a TTSS system and additional proteins encoded on a large virulence plasmid [94–97].

Enteric pathogens cause a variety of diseases in humans but one undeniable symptom is the presentation of gastroenteritis. Some bacterial enteric infections are characterized by disruption of the normal movement of electrolytes and

Table 1: Antimicrobial peptides/proteins and their targets.

Class	Examples	Expression	Action	References
α-defensin	HD-5, HD-6	Paneth cells	*L. monocytogenes*	[38–41]
			E. coli	
			S. typhimurium	
β-defensin	hBD-1	IECs	*P. aeruginosa*	[42–46]
	hBD-2		*E. coli*	
			Candida albicans	
Cathelicidin	hLL37	IECs	*Salmonella*	[47, 48]
Angiogenin	Angiogenin-4	Paneth cells	Gram positive	[49–51]
			Bacteria	
C-type lectin	RegIIIγ	IECs	Gram positive	[52–54]
			Bacteria	

water across the epithelium, which is converted from a state of net fluid absorption to one of net fluid secretion [98]. Secretory diarrhea, as a result of epithelial chloride secretion, has long been regarded as a host defense mechanism. This is based on the notion that increased fluid and electrolyte movement into the gut lumen helps to inhibit adherence of pathogenic organisms by "flushing" them from the body. However, it could also be argued that the induction of pathogen-induced diarrhea is a way to ensure transmission to new hosts, and thus pathogenic fitness [99]. These ideas are not mutually exclusive and secretory diarrhea may be advantageous to both host and pathogen.

Pathogenic bacteria cause diarrhea by multiple mechanisms. *Vibrio cholerae* reside in the lumen of the small intestine and produce toxins, which alter ion absorption and/or secretion [100, 101]. Other bacteria such as *Shigella* and enteroinvasive *E. coli* invade and destroy the colonic epithelium leading to dysentery [102]. More recently pathogenic *E. coli* have been shown to increase chloride ion secretion from intestinal epithelia by upregulating the expression of the receptor for the neuropeptide galanin 1 [103]. Rotavirus, another important cause of diarrhea in infants, induces this condition by activating the enteric nervous system [104, 105].

A large influx of neutrophils (PMNs) into the mucosa and lumen from the underlying vasculature is a significant feature of intestinal bacterial infections [105, 106]. During infection of epithelial cells by enteric pathogens such as *S. typhimurium* and *S. flexneri*, IL-8 is synthesized and secreted baslaterally. Such basolateral IL-8 release imprints subepithelial matrices with long-lived haptotactic gradients that serve to guide neutrophils through the lamina propria to a subepithelial position [107]. However, basolateral IL-8 release is insufficient to induce the migration of neutrophils across the intestinal epithelium, suggesting that the production of other inflammatory mediators, whose release would probably be polarized apically, is important for the execution of this step in the inflammatory pathway [107, 108]. In support of this contention, Kucharzik et al. recently developed a double transgenic mouse model with the ability to induce human IL-8 expression restricted to the intestinal epithelium [109]. The results from this transgenic model showed that although acute induction of IL-8 in the intestinal epithelium is sufficient to trigger neutrophil recruitment to the lamina propria, additional signals are required for neutrophil transepithelial migration and mucosal tissue injury. Indeed, recent evidence suggests that the eicosanoid, hepoxilin A_3, is secreted apically and is responsible for the final step of neutrophil transepithelial migration into the gut lumen [110, 111]. This process is quite complex as distinct signaling pathways mediate *S. typhimurium* invasion, induction of CXCL8 secretion, and induction of hepoxilin A_3 secretion [111–113].

The ability of *Salmonella* serotypes to elicit PMN transmigration in vitro correlates with their ability to cause diffuse enteritis (defined histologically as transepithelial migration of neutrophils), but not typhoid fever in humans [114]. Moreover, large-scale PMN transepithelial migration causes decreased barrier function [115]. Studies exploring the mechanism underlying the release of HXA_3 during infection with *S. typhimurium* revealed the involvement of the *S. typhimurium* type III secreted effector protein, SipA [116]. The *S. typhimurium* effector protein, SipA, promotes a lipid signal transduction cascade that recruits an ADP-ribosylation factor 6 guanine nucleotide exchange factor (such as ARNO) to the apical plasma membrane. ARNO facilitates ADP-ribosylation factor 6 activation at the apical membrane, which in turn, stimulates phospholipase D recruitment to and activity at this site. The phospholipase D product, phosphatidic acid, is metabolized by a phosphohydrolase into diacylglycerol, which recruits cytosolic protein kinase C (PKC)-alpha to the apical membrane. Through a process that is less understood, activated PKC-alpha

phosphorylates downstream targets that are responsible for the production and apical release of HXA_3, which drives transepithelial neutrophil movement [117].

5. PROTOTYPICAL INTERACTIONS BETWEEN PATHOGENIC BACTERIA AND COMMENSAL MICROBIOTA

There are ample lines of evidence to support the emerging concept that a change in the composition of the commensal microbiota alters the intestinal microenvironment making this niche vulnerable to pathogenic insult. In this section we discuss examples to illustrate the remarkable crosstalk between the host, its intestinal microbiota, and potential pathogenic bacteria.

It has been well documented that *S. typhimurium* causes a systemic (typhoid fever) infection in mice while in humans this enteric pathogen causes gastroenteritis. However, Barthel et al. discovered that pretreatment of C57BL/6 mice with streptomycin, an antibiotic that kills facultative anaerobes, followed by infection with a streptomycin-resistant strain of *S. typhimurium* produced a robust intestinal inflammatory response [118]. Such enteritis is primarily characterized by inflammation in the cecum, and also presents with several of the typical pathological hallmarks of acute *Salmonella*-induced gastroenteritis in humans, including PMN infiltration and epithelial cell erosion. This is an intriguing result since the only difference between the untreated and streptomycin treated mice is the alteration of the commensal flora; thus, demonstrating that the presence of the microflora plays a protective role against pathogenic invaders. This study also substantiates the long-standing finding of Barrow and Tucker who found that pretreatment of a chicken's cecum with three different strains of *E. coli* significantly reduced infection with *Salmonella* as compared to untreated animals [119]. Additionally, Hudault et al. (2001) determined that the presence of a single species of *E. coli* in the gut could restrict the infection of S . *typhimurium* as compared to its germ-free counterpart [120].

More recently, Stecher et al. used the *S. typhimurium* colitis model to investigate competition between an enteric pathogen and the host microbiota [121]. This group found that inflammatory responses induced by *S. typhimurium* led to profound perturbations in the composition of the commensal microbiota as determined by 16S rRNA. The inflammatory host responses induced by *S. typhimurium* not only changed the microbiota composition but also suppressed its growth, thereby, overcoming colonization resistance. In contrast, an avirulent *Salmonella* mutant defective in triggering inflammation was unable to overcome colonization resistance. These results raise an interesting point in that perhaps the intestinal inflammation induced by *S. typhimurium* might be a crucial event in order to overcome colonization resistance. In this respect, triggering the host's immune defense may shift the balance between the protective microbiota and the pathogen to favor the pathogen. The idea that the intestinal microbiota can be altered by invading pathogens is further supported by Lupp et al. who found that host-mediated inflammation in response to an infectious agent induced alterations in the colonic community that not only resulted in the elimination of a subset of indigenous microbiota but also led to the growth of the Enterobacteriaceae family [122]. Moreover, in children undergoing treatment for diarrhea, fluctuations in the intestinal microflora were observed for both rotaviral and nonrotaviral-induced diarrhea [123]. This phenotype was reversed and the normal microflora was re-established after about three months of the disease episode. Other studies have investigated the role of the intestinal microbiota during infectious disease transmission. In particular, Lawley et al. describe a model in which persistently infected 129X1/SvJ mice provide a natural model of transmission. In this model, only a subset of mice termed "supershedders" could shed high levels of bacteria in their feces. Whereas immunosuppression of the infected mice did not induce the supershedder phenotype, antibiotic treated mice displayed a high supershedder phenotype [124]. Together, these studies suggest that the intestinal microbiota plays a critical role in controlling pathogen infection, disease, and even transmissibility.

There are also examples in which members of the commensal microflora are able to cause disease. This is specifically illustrated by *Enterococcus faecalis*, a prominent member of the GI tract microbiota. In a healthy intestine these bacteria behave as a normal resident of the intestinal ecosystem. However, in individuals undergoing antibiotic treatment or those who are immunocompromised, *E. faecalis* is able to colonize new niches of the intestinal microenvironment as a certain subgroup of this species is antibiotic resistant (Figure 1(c)). Under such compromised conditions, *E. faecalis* can infect and spread to other sites of the host such as the bloodstream, urinary tract, and surgical wounds. Not surprisingly, the subgroup population harboring the antibiotic resistance genes also has genetic elements conferring infectivity and virulence. Furthermore, the genome sequence of *E. faecalis* strain V583, the most causative agent of vancomycin resistant enterococcal infection in America, [125] was recently reported [126]. Recent studies have determined that more than 25% of the *E. faecalis* genome is most likely derived from mobile or foreign DNA, which might have contributed to the rapid acquisition and dissemination of drug resistant strains [126]. Another example is illustrated by *Clostridium difficle*, a Gram-positive bacterium that can harmlessly inhabit the human intestine. However, certain individuals undergoing antibiotic therapy, as a result of their altered intestinal microflora, presented with *C. difficle* infection accompanied with severe intestinal colitis (Figure 1(c)) [127].

Commensal bacteria, such as *Bacteroides fragilis*, may also inhibit other opportunistic members of the intestinal microflora from causing disease [128]. *B. fragilis* is a Gram-negative bacterium that resides in a healthy human intestine. Normally, this bacterium expresses a surface carbohydrate capsule known as polysaccharide A (PSA), which contributes to many beneficial activities underlying the immune development of the host, including activation of CD4+ T cells, and stimulation of the innate immune responses through TLR2 signaling. Mazmanian et al. determined that *B. fragilis* protects the host from *Helicobacter hepaticus*-induced

colitis in experimental mice. However, in animals harboring *B. fragilis* strains that do not express PSA, *H. hepaticus* colonization led to disease and production of proinflammatory cytokines induced by intestinal immune cells [128, 129]. Thus, in healthy individuals it appears that PSA from *B. fragilis* is necessary to confer some beneficial activity. In spite of this, PSA was also found to potentiate the ability of *B. fragilis* to cause disease in patients who have a compromised mucosal surface, such as postsurgical patients. This function is initiated upon submucosal entry of the bacteria during which PSA activates CD4+ T cells leading to abscess formation [130].

6. ROLE OF BACTERIA IN INFLAMMATORY BOWEL DISEASE

Recent evidence from a variety of investigative avenues implicates abnormal host-microbial interactions in the pathogenesis of inflammatory bowel disease (IBD). In fact, IBDs preferentially occur in the colon and distal ileum (i.e., locations that contain the highest concentrations of intestinal bacteria). An important role for microbial agents in the pathogenesis of IBD is inferred by numerous recent studies, which conclude the bacterial flora differs between patients with inflammatory bowel disease (IBD) and healthy individuals. Moreover, accumulating evidence suggests that the composition and function of the microbiota in patients suffering with IBD are abnormal.

Ninety-nine percent of the gut microbiota in healthy individuals is composed of species within four bacterial divisions: *Firmicutes*, *Bacteroidetes*, *Proeobacteia*, and *Actinobacteria* [131, 132]. Investigation of the microbial diversity in active IBD is a highly pursued topic of interest and is an area of research still at its infancy. In IBD patients, early returns have suggested that there is a decrease in the number of beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus* spp., and an increase in pathogenic bacteria, such as a *Bacteroides* and *Escherichia coli* [132–136]. Such dysbiosis induces a breakdown in the balance between putative spp. of protective versus harmful bacteria, and may promote inflammation. Other studies have shown that there is a decrease in microbial diversity that accompanies the increased numbers of Enterobacteriaceae, including *E. coli*, with decreased numbers of *Firmicutes*, and a particular decrease in *Clostridium* species. As convincing as this data is, there is still a lack of evidence to denote whether a specific pathogen is responsible for onsets or relapses of IBD [132]. Further, the most compelling studies are derived from animal models. Regardless, a number of organisms have been implicated in Crohn's disease, with *Mycobacterium paratuberculosis* and *E. coli* drawing a great deal of attention [137].

Patients with IBD have higher numbers of mucosa-associated bacteria than control patients [138], and the generalized or local dysbiosis observed is due to the presence of low numbers of normal bacteria and high numbers of unusual bacteria with a decrease in biodiversity. The composition of the increased numbers of bacteria attached to the intestinal epithelium of IBD patients are from diverse genera. *Bacteroides spp*., in particular, has been identified as a predominate member of the epithelial layer, and in some instances was located intracellularly [136]. While this remains an intriguing observation, the role of *Bacteroides* in IBD is still unclear. Furthermore, distinct adherent or invasive *E. coli* has been identified in the ileal mucus of patients with Crohn's disease, and the involvement of a new potentially pathogenic group of adherent invasive *E. coli* (AIEC) has been suggested [139]. For instance, in studies aimed to assess the predominance of *E. coli* strains associated with the ileal mucosa of Crohn's disease patients, *E. coli* was recovered from 65% of chronic lesions and from 100% of the biopsies of early lesions. By comparison, 3–6% of the *E. coli* was recovered form healthy ileal mucosa. *E. coli* was also abnormally present (50–100% of the total number of aerobes and anaerobes) in early and chronic ileal lesions of CD patients [140, 141]. These observations were confirmed in a subsequent study in which adherent *E. coli* was found in 38% of patients with active ileal Crohn's disease [133]. This study also revealed that the number of *E. coli* in situ correlated with the severity of the disease, and that the invasive *E. coli* was also restricted to the inflamed mucosa. Interestingly, the recovered *E. coli* strains were predominantly novel in phylogeny, displayed pathogen-like behavior in vitro, and expressed virulence factors [133].

It is suspected that the abnormal colonization of the lieal mucosa is largely due to increased expression of CEACAM6, a receptor for adherent-invasive *E. coli* [142]. However, Crohn's disease patients also exhibit defective microbial killing mechanisms that result in increased exposure to commensal bacteria. For example, Crohn's disease patients have defective antimicrobial peptide production, including α-defensin 5 in ileal disease and human β-defensin 2 in Crohn's colitis [143, 144]. This is accompanied by functional abnormalities in the killing of *Bacteroides vulgatus*, *E. coli*, and *Enterococcus faecalis* [145]. In addition, NOD2 polymorphisms in Crohn's disease are associated with selective decrease in α-defensin production by Paneth cells, as well as in defective clearance of intracellular pathogens by colonic epithelial cells [146]. Thus, combined with defective antimicrobial peptide function in Crohn's disease the functional changes described above provide a reasonable rationale for the profound increase in mucosally associated Enterobacteriaceae. Also, in light of the alteration in the composition of the luminal microbiota, it is perhaps not surprising that Crohn's disease has features that might be the consequence of a microbial process. This is exemplified by the noted infection of Peyer's patches and lymphoid aggregates, and the presence of ulcerations, microabscesses, fissures, fistulas, granulomas, and lymphangitis [137].

As evidence accumulates to suggest that dysbiosis in IBD patients induces a breakdown in the balance between putative spp. of protective versus harmful bacteria, one potential new method of intervention lies in the modulation of the enteric flora. Indeed, current studies suggest that probiotics might offer an alternative or adjuvant approach to conventional IBD therapies by altering the intestinal microflora and, in turn, modulating the host immune system. Probiotics are defined as living food supplements

or components of bacteria that have a beneficial effect on human health. Indeed, probiotic activity has been associated with *Lactobacillus*, *Bifidobacteria*, *Streptococcus*, *Enterococcus*, nonpathogenic *E. coli*, and *Saccharomyces bourlardii* [147, 148].

Probiotic supplements may balance the indigenous microflora in IBD patients. A growing body of literature supports this emerging concept, which suggests that probiotics have therapeutic effects in ulcerative colitis, Crohn's disease and pouchitis [147, 148]. The rationale for employing probiotics in the treatment of IBD is underscored by the proposed pathogenic role of the intestinal microflora in this disease. Numerous studies support the notion that introduction of probiotics to the GI tract can alter the enteric microflora in IBD patients, which in turn has a profound effect on intestinal defense mechanisms, including (i) inhibiting microbial pathogenic growth, (ii) increasing epithelial cell tight junctions and permeability, (iii) modulating the immune response of the intestinal mucosa, (iv) increasing the secretion of antimicrobial products, and (iv) eliminating pathogenic antigens [149–151]. Thus, such broad mechanistic effects of probiotics may explain the beneficial effects observed.

Probiotic preparations are primarily based on a variety of lactic acid bacteria (lactobacilli, bifidobacteria, and streptococci), which under healthy conditions are normal and important components of the commensal microbiota. In addition, probiotic mixtures often contain some nonpathogenic bacteria that include *E. coli*, enterococci, or yeast (*Saccharomyces bourlardii*) [152]. Probiotic strains also need to satisfy important criteria. First, probiotics must be safe and tested for human use [149, 152]. In addition, such strains should be of human origin, resistant to acid and bile, and survive and be metabolically active within the intestinal lumen. Probiotics must also be antagonistic against pathogenic bacteria as they produce antimicrobial substances, compete within the GI tract, and promote a reduction in colonic pH.

Many clinical trials have documented that probiotics can achieve and maintain remission in patients with ulcerative colitis, and also prevent and maintain remission of pouchitis. However, probiotics seem to be ineffective in Crohn's disease [153]. Although controlled clinical trials are still required to investigate the unresolved issues related to efficacy, dose, duration of use, single or multistrain formulation, and simultaneous use of probiotics, synbiotics, or antibiotics, the preliminary data for the therapeutic use of probiotics in selective patients with mild to moderate IBD are encouraging.

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Review Article

Application of Sequence-Dependent Electrophoresis Fingerprinting in Exploring Biodiversity and Population Dynamics of Human Intestinal Microbiota: What Can Be Revealed?

Geert Huys, Tom Vanhoutte, and Peter Vandamme

Laboratory of Microbiology, Faculty of Sciences, Ghent University, K.L. Ledeganckstraat 35, 9000 Ghent, Belgium

Correspondence should be addressed to Geert Huys, geert.huys@ugent.be

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Sequence-dependent electrophoresis (SDE) fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) have become commonplace in the field of molecular microbial ecology. The success of the SDE technology lays in the fact that it allows visualization of the predominant members of complex microbial ecosystems independent of their culturability and without prior knowledge on the complexity and diversity of the ecosystem. Mainly using the prokaryotic 16S rRNA gene as PCR amplification target, SDE-based community fingerprinting turned into one of the leading molecular tools to unravel the diversity and population dynamics of human intestinal microbiota. The first part of this review covers the methodological concept of SDE fingerprinting and the technical hurdles for analyzing intestinal samples. Subsequently, the current state-of-the-art of DGGE and related techniques to analyze human intestinal microbiota from healthy individuals and from patients with intestinal disorders is surveyed. In addition, the applicability of SDE analysis to monitor intestinal population changes upon nutritional or therapeutic interventions is critically evaluated.

1. INTRODUCTION

The mammalian intestinal tract comprises a highly complex population of microorganisms reaching up to 10^{14} bacteria in the large intestine [1]. Starting off as a sterile system at birth, microbial colonization of the intestine develops in a successive manner in which bacteria predominate along with lower numbers of archae, yeasts, filamentous fungi, parasites, and viruses [2, 3]. Following initial domination by facultative anaerobes, the gut microbiota becomes gradually inhabited by obligate anaerobes which will remain its major constituents during adult life [4–7]. Triggered by the growing number of 16S ribosomal RNA (rRNA)-based approaches, insights in the evolutionary diversity of the human adult gut flora has changed drastically in recent years. Based on a delineation level of 98% 16S rRNA gene sequence similarity, current estimates indicate that the human gastrointestinal tract encompasses more than 1000 bacterial phylogenetic types, also referred to as phylotypes or "molecular species" [8–10]. These taxonomic inventory studies have revealed that the gut microbiota in adults is largely dominated by members of only two bacterial phyla, that is, the Bacteroidetes and the Firmicutes, and one member of the archaea, *Methanobrevibacter smithii*. Through a complex network of mutualistic interactions, the gut microbiota has a profound impact on the host's health by acting as a barrier against pathogens, contributing to the degradation of food components, stimulating the host immune system, and producing a series of essential vitamins, enzymes, and short-chain fatty acids [11–14].

Until a decade ago, knowledge on the taxonomic composition and metabolic activity of the intestinal tract microbiota was mainly based on the use of culture-dependent techniques. Triggered by the growing awareness that only a fraction of the gut microbiota is culturable under laboratory conditions, various culture-independent methods

have been evaluated in intestinal microbial ecology [15–19]. Depending on the scientific rationale and technical design of the study, molecular approaches for assessing diversity and dynamics of intestinal microbiota include population fingerprinting [this review], clone libraries [20–23], dot blot hybridization [24, 25], fluorescent in situ hybridization (FISH) [8, 26–29], real-time PCR [30–33], DNA microarrays [34–36], and metagenomics [9, 37–39].

In contrast to several of the aforementioned techniques that specifically target one or more autochthonous members of intestinal tract or that require analysis of large and complex datasets, population fingerprinting is a universal concept that allows one to characterize and monitor intestinal microbiota without preexisting knowledge of its structure or composition. The most commonly used fingerprinting techniques in the field of intestinal microbiology are based on the sequence-dependent electrophoresis (SDE) principle and include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), and temporal temperature gradient gel electrophoresis (TTGE). In contrast to conventional gel electrophoresis based on fragment size, the SDE principle relies on the sequence-dependent electrophoretic separation of a mixture of equally sized PCR products in a polyacrylamide gel containing a linear gradient of chemical denaturants (DGGE) or a linear temperature gradient (TGGE and TTGE). This way, separation is achieved by the gradually decreasing electrophoretic mobility of partially melted, double-stranded amplicons in the denaturing gradient. PCR fragments equal in length but with different sequences have a different melting behavior and will stop migrating at different positions along the gel, eventually producing a banding pattern or fingerprint. To a lesser extent, also single-strand conformation polymorphism (SSCP) [40] and terminal-restriction fragment length polymorphism (T-RFLP) [41] analysis have been applied in microbial community profiling. Likewise SDE methods, both these methods rely on PCR amplification of specific target sequences followed by electrophoretic separation of amplicons. Whereas this separation is based on sequence-specific melting behavior of amplicons in SDE analysis, the taxonomic resolution of SSCP and T-RFLP is determined by the secondary structure of ssDNA or by the distribution of endonuclease restriction sites, respectively. The principle of SSCP analysis is essentially based on the sequence-dependent differential intramolecular folding of ssDNA which alters the migration speed of the molecules [42]. The ssDNA fragments originating from PCR amplicons are separated using uniform, low temperature, nondenaturing electrophoresis to maintain the secondary structure of the single-stranded fragments. T-RFLP analysis, on the other hand, is based on a size-dependent electrophoretic separation of digested fluorescently end-labeled PCR products. Upon electrophoresis using either gel- or capillary-based systems, only the "terminal" end-labeled restriction fragments are detected. Although less commonly used than DGGE and related techniques, SSCP [43] and, especially, T-RFLP [44–48] have been applied to study the diversity and dynamics of intestinal microbiota. This review will specifically focus on the use of SDE techniques, and DGGE in particular, in the field of intestinal microbiology.

Since their introduction in microbial ecology in the early 1990s [49], SDE fingerprinting techniques have been employed to analyze microbial communities in a wide range of environments including aquatic sites [50–52], soil [53], fermented foods [54, 55], and the human intestinal tract [this review]. The value of SDE-based fingerprinting methods in intestinal microbiology lays in the fact that they allow pattern-based visualization of the predominant bacterial groups including poorly culturable and currently uncultured bacteria that are considered to represent up to 50–90% of the intestinal microbiota.

This review will deal with all different aspects of SDE methodology including its possibilities and limitations in terms of reproducibility, sensitivity, and data analysis. Through discussion of selected studies that have contributed to the field, an overview will be presented of SDE-based research approaches to study human intestinal ecosystems in relation to the microbial ecology of healthy and disease-affected populations. The scope of this review excludes SDE applications dealing with the human upper gastrointestinal tract or with animal intestinal ecosystems.

2. METHODOLOGY

2.1. Principle

The principle of SDE techniques relies on the electrophoretic separation of PCR amplicons with equal length in a sequence-specific manner in a polyacrylamide matrix containing a defined denaturing gradient of urea and formamide (DGGE) or temperature gradient (TGGE and TTGE). The temperature gradient in TGGE is created along the length of the gel, whereas in TTGE a temporal temperature gradient is gradually formed during the electrophoresis run. The electrophoretic mobility of double-stranded amplicons in a gel matrix with an increasing denaturing gradient is retarded at a given chemical denaturant concentration or temperature that causes (partial) melting of the sequence region with lowest melting temperature (T_m). The physical denaturation of the dsDNA fragment is thus largely determined by its nucleotide sequence and %G+C content and proceeds in discrete portions of the fragment or the so-called melting domains. These domains interfere with the helical structure of the DNA molecule and will eventually halt further migration. Amplicons that are different at the sequence level are likely to display a different melting behavior and will, therefore, stop migrating at different positions along the linear gradient of the gel, which upon visualization will result in band profiles representing the sequence diversity of the amplicon mixture.

In practice, SDE-based community profiling comprises four steps: (i) extraction of total community DNA from the sample; (ii) PCR-controlled amplification using specific oligonucleotide primers; (iii) sequence-dependent electrophoretic separation of the amplicons using either DGGE, TGGE or TTGE; and (iv) fingerprint processing and analysis.

2.2. Sampling and total DNA extraction

2.2.1. Sample collection and processing

The endogenous microbiota differs along the length of the intestinal tract [56]. In addition to the longitudinal diversity gradient, also a cross-sectional differentiation of the microbial population has been observed in the lumen, the mucosa, and the epithelium surface [56, 57]. Mainly due to sampling difficulties, the taxonomic composition of these microhabitats in the intestinal tract is poorly documented. Because of this spatial distribution, microbiological data obtained from a subsample of the gut cannot always be extrapolated to the global composition of the entire intestinal microbiota. Most often, fecal samples are used to study the intestinal microbiota because they are the most accessible type of specimen that can be collected from this environment. In specific clinical cases, also luminal endoscopy samples, mucus, biopsies, and stoma liquid can be analyzed.

In most studies, immediate processing of samples is not feasible due to the need for transportation and/or (long-term) storage of the specimen. It has been shown that storage of stool samples at room temperature and even at 4°C showed a substantial reduction in bacterial diversity and the degradation of bacterial DNA after 8 hours [58]. Therefore, it is generally recommended that colon samples should be (deep-)frozen immediately upon collection and stored at maximally −20°C and preferably at −70°C, until further processing. However, it should be kept in mind that repeated freezing and thawing of samples can have negative effects on bacterial viability and recovery rates [59–61]. Although poorly documented, the impact of subsequent sample manipulations on DNA extraction and the yield and quality of the resulting DNA probably is less dramatic [62].

2.2.2. Extraction of community DNA

An efficient, reproducible, and high-yield method for total DNA extraction is indispensable in order to obtain a representative view of the actual microbial composition of an intestinal sample. The most crucial step in any DNA extraction procedure is cell lysis. A series of methods including commercial kits and inhouse laboratory protocols have been described and evaluated for the extraction of total bacterial DNA or RNA from intestinal samples making use of chemical, mechanical (e.g., beads), and/or enzymatic lysis [63–67]. Because not all members of the intestinal microbiota display the same sensitivity to the lysis conditions of a given procedure, it is extremely difficult, if not impossible, to extract DNA from all constituting species with the same efficiency. Furthermore, the DNA isolation procedure should also be able to remove potential PCR inhibitors that may be present in fecal samples such as phenols, bile salts, degradation products of hemoglobin, and complex polysaccharides of plant origin. The selection criteria usually applied to evaluate the efficacy of a DNA extraction method include electrophoretic verification of DNA integrity, determination of DNA yield and quality using spectrophotometric analysis and quality control of the obtained SDE profile [64–66]. In addition, the lysis efficiency of different DNA extraction protocols can be compared based on the complexity and band intensity of SDE community fingerprints. Upon extraction, DNA solutions are generally stored at −20°C. The influence of storage conditions and duration of storage on the integrity and quality of total DNA extracts from intestinal samples has not been studied in great detail.

Depending on the type of (clinical) application, high-quality community DNA may need to be obtained from a range of different sample types such as digesta, mucosal, and fecal samples. For this reason, the DNA extraction technique should be carefully selected and possibly further evaluated or optimized with particular attention for the type and number of specimens [66]. In this respect, it may be less appropriate to use commercial DNA extraction kits given the limited possibilities to optimize the procedure, for example, by changing concentrations or composition of extraction reagents. On the other hand, commercial kits can considerably reduce the hands on time compared to more complex inhouse protocols. In Table 1, technical details are given for a number of frequently used total DNA extraction procedures that have been used in SDE-based profiling of human intestinal microbial communities [6, 21, 24, 32, 33, 43, 63–66, 68–109].

2.3. Community PCR

Following DNA extraction and purification, multiple primer sets with different taxonomic coverage can be applied for community PCR amplification. The use of universal PCR primers allows any microbial community to be analyzed, although in ecosystems with a high diversity like the intestinal tract only the (pre)dominant constituents will in effect generate a visible band in SDE. In order to focus on a specific subpopulation within the total community, group-specific PCR primers can be used which allow detection of bacterial taxa that are less prevalent in the intestinal tract. Traditionally, universal and specific community PCR primers for SDE applications are designed using the 16S rRNA gene as a target molecule. This preference stems from the fact that the SSU rRNA gene has a mosaic structure composed of both invariant, relatively conserved, and highly variable regions (V regions). In SDE-based population fingerprinting, primers are used that anneal to conserved sequence parts of the gene in order to cover one up to three hypervariable regions. In Table 2, a selection is presented of universal and specific primer sets that have been used in SDE-based profiling of human intestinal microbial communities [6, 30, 64, 65, 69, 71–74, 76, 77, 79–82, 84–86, 88, 90, 91, 94–96, 98, 99, 101, 102, 105–130]. Taking the rumen as model system of complex microbial community, Yu and Morrison [131] systematically compared a set of DGGE profiles obtained with universal primers targeting different V regions. Based on sequence variability and temperature heterogeneity of the lowest T_m domain of the V region and on the number, resolution, and relative intensity of the bands in the resulting DGGE profile, the V3 region was most preferred for analyzing intestinal microbiomes. In addition,

Table 1: DNA extraction procedures used in SDE-based profiling of human intestinal microbial communities.

Description or reference	Sample type	Cell lysis (reagents or principle)	DNA extraction	Application(s)	Selected reference(s)
FastDNA kit (Bio101 Carlsbad, Calif, USA)[a] FastDNA SPIN kit (Qbiogene, Carlsbad, Calif, USA)[a]	Feces; mucosa biopsies	Chemical (guanidium salts and detergents) and mechanical (bead beating using garnet mix)	Silica-based binding matrix (and spin filters)[a]	DGGE; SSCP; real-time PCR; cloning; sequencing	[43, 63, 68–76]
QIAampDNA Stool Mini Kit (Qiagen, Valencia, Calif, USA)	Feces; mucosa biopsies	Chemical (guanidium salts and detergents)	Silica-gel membrane spin columns	DGGE; TGGE; real-time PCR; cloning; sequencing	[63, 77–86]
Modified protocol of [87]	Feces	Enzymatic (lysozyme and mutanolysin) and chemical-enzymatic (SDS and proteinase K)	Phenol-chloroform-isoamylalcohol and chloroform	DGGE; sequencing	[88]
Modified protocol of [89]	Feces	Enzymatic (lysozyme and mutanolysin) and chemical (guanidiumthiocyanate-EDTA-sarkosyl)	Chloroform-isoamylalcohol	DGGE; real-time PCR	[64, 90]
[24]	Feces; mucosa biopsies	Mechanical (bead beating)	TTGE	[91–94]	
[95]	Feces; cecal fluids; mucosa biopsies	Mechanical (bead beating in acid phenol)	Phenol-chloroform and chloroform	DGGE; TGGE; cloning; sequencing	[6, 65, 66, 96–102]
[32]	Feces	Mechanical-chemical (bead beating in buffer-saturated phenol and SDS)	Phenol-chloroform	Group-specific PCR; real-time PCR	[33]
[103]	Feces	Chemical (guanidiumthiocyanate and sarkosyl) and mechanical (bead beating)	Polyvinyl-polypyrrolidone	TTGE; cloning; sequencing	[21, 104–109][b]

[a] FastDNA kit (Bio101) and FastDNA SPIN Kit (Qbiogene) only differ in the use of spin filters during the silica-DNA purification.
[b] The authors of [106–108] reported a modified protocol of [103] in which also phenol and chloroform-isoamylalcohol were applied in the extraction procedure.

the authors recommended to use the V3–V5 or V6–V8 regions if a longer amplicon is preferred. Next to the SSU rRNA gene, also its rRNA counterpart can be coextracted and used as PCR template in SDE analyses of intestinal ecosystems when preceded by reverse transcription [70, 71, 75, 93, 95]. In this way, SDE profiles are generated that represent the (pre)dominant metabolically active bacteria based on the assumption that the cells of these organisms generally have a much higher ribosomal RNA content and rRNA/DNA ratio compared to resting cells.

An additional 40-nucleotide GC rich sequence, the so-called GC-clamp, is usually attached to the 5′ end of one or both of the PCR primers and participates in the PCR reaction. This way, the GC-tail generated at the end of the amplicon will prevent complete denaturation of the product and is necessary to obtain a stable melting behavior of the fragments during electrophoresis [49, 132, 133]. GC-clamps can vary in sequence, length, and location [100, 134–136], and their design needs to be based on the target sequence and the primers used. Mutation analysis data have shown that GC-clamps have the strongest effect on the melting properties of short fragments (<300 bp) and that this effect may be drastically reduced for large fragments (>400 bp) [136]. Also, it has been demonstrated that a GC-clamp length of 60 bp may be efficient for detection of fragments with a T_m value close to 80°C whereas fragments with $T_m > 80$°C may require longer GC-clamps in combination with naturally occurring high-melting (thus GC-rich) domains [136].

2.4. Sequence-dependent electrophoresis

2.4.1. Electrophoresis conditions

Essentially, a DGGE system consists of a heated buffer tank operated under strict control of temperature and stable buffer circulation. Several systems are currently available, of which DCode (Bio-Rad Laboratories; http://www.bio-rad.com/), INGENYphorU (Ingeny; http://www.ingeny.com/),

Table 2: Universal and group-specific PCR primers used in SDE-based profiling of human intestinal microbial communities.

Target group(s)	Primer designation	Sequence (5′-3′)[a]	Target region	Selected reference(s)
		Domain level		
Bacteria	HDA1[b]	ACTCCTACGGGAGGCAGCAGT	V2-V3-16S rDNA	[30, 76, 102, 110–114]
	HDA2[b]	GTATTACCGCGGCTGCTGGCAC		
	F357	CCTACGGGAGGCAGCAG	V3-16S rDNA	[64, 72, 73, 77, 79, 115–117]
	518R	ATTACCGCGGCTGCTGG		
	339F[c]	CTCCTACGGGAGGCAGCAG	V3-V4-16S rDNA	[94, 106, 107]
	788R	GGACTACCAGGGTATCTAA		
	U968-F	AACGCGAAGAACCTTAC	V6–V8-16S rDNA	[6, 64, 65, 69, 71, 79, 80, 82, 84, 85, 91, 95, 96, 101, 105, 109, 117–123]
	L1401-R	CGGTGTGTACAAGACCC		
		Genus (group) level		
Bacteroides	FD1	AGAGTTTGATCCTGGCTCAG	16S rDNA	[124]
	RbacPre	TCACCGTTGCCGGCGTACTC		
	Bfr-F	CTGAACCAGCCAAGTAGCG	16S rDNA	[81]
	Bfr-R	CCGCAAACTTTCACAACTGACTTA		
Bifidobacterium	Bif164-f	GGGTGGTAATGCCGGATG	16S rDNA	[69, 79, 82, 85, 99, 101, 105, 108, 118, 125–127]
	Bif662-r	CCACCGTTACACCGGGAA		
	g-Bifid-F	CTCCTGGAAACGGGTGG	16S rDNA	[64]
	g-Bifid-R	GGTGTTCTTCCCGATATCTACA		
	ForTal	CGTCGCCTTCTTCTTCGTCTC	transaldolase gene	[74]
	RevTal	CTTCTCCGGCATGGTGTTGAC		
Helicobacter	658f	TGGGAGAGGTAGGTGGAAT	16S rDNA	[128]
	1067R	GCCGTGCAGCACCTGTTTTCA		
Enterococcus	Ent1017F	CCTTTGACCACTCTAGAG	16S rDNA	[64]
	Ent1263R	CTTAGCCTCGCGACT		
Lactobacillus group[d]	Lac1	AGCAGTAGGGAATCTTCCA	16S rDNA	[64, 86, 88, 125, 127, 129, 130]
	Lac2	ATTYCACCGCTACACATG		
	27f (also Bact-0011f)	AGAGTTTGAT(C/T)(A/C)TGGCTCAG	16S rDNA	[79, 98, 118]
	Lab-0677r	CACCGCTACACATGGAG		
	Lab-0159f	GGAAACAG(A/G)TGCTAATACCG	16S rDNA	[98, 118]
	Uni-0515-r	ATCGTATTACCGCGGCTGCTGGCA		
	Lab-0159f	GGAAACAG(A/G)TGCTAATACCG	16S rDNA	[124]
	Lab-0677r	CACCGCTACACATGGAG		
		Species group level		
Bacteroides fragilis subgroup[e]	g-Bact-F	ATAGCCTTTCGAAAGRAAGAT	16S rDNA	[73]
	g-Bact-R	CCAGTATCAACTGCAATTTTA		
	Bact 596F	TCAGTTGTGAAAGTTTGCG	16S rDNA	[64]
	Bact 826R	GTRTATCGCMAACAGCGA		
	Bact 531F	ATACGGAGGATCCGAGCGTTA	16S rDNA	[90]
	Bact 766R	CTGTTTGATACCCACACT		
Clostridium phylogenetic clusters XI and XIVa[f]	Erec 688F	GCGTAGATATTAGGAGGAAC	16S rDNA	[90]
	Erec 841R	TGCGTTWGCKRCGGCACCG		

[a] A GC-clamp is attached to the 5′ end of either the forward or reverse primer.
[b] Primers HDA1 and HDA2 have the same core sequence as primers 341 f and 518 r, respectively, but with a few additional nucleotides at both 5′ and 3′ ends.
[c] Primer 339f has the same core sequence as primer 341 f but with two additional nucleotides at the 5′ end.
[d] The *Lactobacillus* group comprising the genera *Lactobacillus, Leuconostoc, Pediococcus, Weissella*, and *Aerococcus* (the latter genus was originally not described as target of the Lac1/2 primers).
[e] The *Bacteroides fragilis* subgroup comprising *B. fragilis, B. acidifaciens, B. caccae, B. eggerthii, B. ovatus, B. stercoris, B. thetaiotaomicron, B. uniformis*, and *B. vulgatus*.
[f] *Clostridium* phylogenetic cluster XI represents the *Clostridium lituseburense* group, whereas *Clostridium* phylogenetic cluster XIVa represents the *Clostridium coccoides-Eubacterium rectale* group.

and DGGEK-1001/2001/2401/4001/4801 (CBS Scientific; http://www.cbsscientific.com/) appear to be most commonly used. The apparatus provided by Bio-Rad and Ingeny can also be applied for TTGE analysis whereas for TGGE a temperature gradient block should be integrated in the system. In case a high number of samples need to be analyzed such as in monitoring studies, the sample capacity of the system is an important criterion. The maximum capacity per run for the three aforementioned systems varies from 60 (DCode), 96 (INGENYphorU) to 128 (DGGEK-4801) samples.

In general, DGGE makes use of parallel gel electrophoretic systems that have an increasing vertical gradient of denaturants parallel to the direction of electrophoresis. In many studies, the optimal denaturing gradient yielding the highest resolution is first determined by perpendicular gradient gels. For this purpose, one sample containing one or more PCR fragments is electrophoretically separated across a denaturing gradient perpendicular to the direction of the electric field resulting in sigmoid-shaped curves. From these gels, the intermediate range of denaturant concentration, where different electrophoretic mobilities between PCR products are obtained, is considered the optimal gradient of denaturants for multilane analysis in parallel DGGE. The optimal time of electrophoresis can be determined through a "time travel" experiment during which a mixture of PCR fragments is loaded onto a parallel gel at constant time intervals. The optimal duration of a DGGE run can be derived from the time needed to obtain maximal separation of amplicons.

A detailed procedure to cast and run DGGE gels has been described by Muyzer et al. [134, 137]. Essentially, the desired low and high concentration of denaturing solution is obtained by mixing zero (0%) and high-concentration (80–100%) denaturing acrylamide solutions in appropriate ratios. Upon the addition of ammoniumpersulphate and tetramethylethylenediamine, the mixture is poured between two vertical glass plates in order to generate a linear denaturing gradient. The concentration of acrylamide usually ranges from 6–12% and depends on the size range of the fragments to be separated. In general, the high-concentration denaturing solution contains 7-8 M urea and 20–40% formamide. Electrophoresis is mostly carried out in 0.5× or 1× TAE-buffer at a fixed voltage between 50 V and 250 V and a constant temperature between 55 and 65°C. Run times generally range from 3–17 hours, although longer run times with lower voltages tend to produce better quality gels.

In the case of TGGE and TTGE, a linearly increasing temperature gradient parallel to the electrophoresis direction or formed during the length of electrophoresis, respectively, is applied in combination with a uniform, high-denaturant polyacrylamide gel to separate PCR fragments. To determine the temperature range for parallel TGGE or TTGE analysis, a melting profile of the DNA sequence can be generated using specialized software (e.g., Poland analysis software; http://www.biophys.uni-duesseldorf.de/local/POLAND/poland.html). The optimal temperature gradient is theoretically delineated by the lowest and highest T_m values obtained in the melting profile. The theoretical T_m values can be lowered by adding denaturing components to the gel, for example, one mole of urea will lower the theoretical T_m with 2°C [138, 139]. In general, a 6–8 M urea gel is used in combination with a typical temperature range between 35 and 70°C.

Unlike many other fingerprinting methods that make use of commercially available size standards, SDE techniques suffer from a lack of consensus regarding standards for normalization. Because denaturing gradients can slightly vary between different gels, a standard reference composed of amplicons from pure cultures that spans a maximal range of the applied gradient should be routinely included at several fixed internal positions on every gel to allow data normalization and gel-to-gel comparison with a high degree of confidence. Neufeld and Mohn [140] proposed an approach which facilitated and improved normalization of samples from multiple gels by including standards in each lane instead of using interlane standards. These intralane standards contain fluorescent tags incorporated in the primers that excitate at another wavelength than that of the fluorescent molecules attached to the unknown PCR product. Furthermore, the application of fluorophore-labeled primers does not require gel staining following electrophoresis, which improves the overall sensitivity of the population fingerprinting procedure and enables additional DGGE versatility including simultaneous analysis of DNA- and RNA-derived mixtures in the same lane.

2.4.2. Gel staining

Upon electrophoresis, gels are stained and digitally captured for further analysis. Three staining agents are commonly used to visualize fragments. Originally, SDE gels were stained with ethidium bromide (EtBr) given its widespread use as an intercalating fluorescent dye used to detect nucleic acids. The next generation of fluorescent nucleic acid dyes such as SYBR Green and similar stains offer an increased sensitivity compared to EtBr due to a lower overall background signal allowing detection of DNA fragments at lower concentrations [64, 134, 137]. Additional advantages of these newer dyes are that they are generally considered to be less toxic or mutagenic than EtBr and can be excited by wavelengths above 400 nm which enables the use of non-UV illumination. One specific member of the SYBR Green family, SYBR Gold, binds to both dsDNA and ssDNA. This specific feature may further enhance the detection sensitivity since DNA amplicons in the SDE gels are partially single stranded. Although less commonly applied, silver staining is generally considered the most sensitive staining procedure. Following DNA fixation with ethanol and acid (e.g., nitric acid), Ag^+ ions in silver nitrate are selectively reduced under alkaline conditions by formaldehyde to metallic silver (Ag) that is visualized as a black precipitate. Potential drawbacks of this procedure include the fact that silver stained gels impede subsequent blotting experiments or band sequence analysis and the aspecific detection of protein components such as BSA and *Taq* polymerase present in the PCR mix which may generate additional background signals [134, 137].

2.5. Data analysis

Normalized SDE fingerprints can be analyzed visually and/or numerically. Visual interpretation is attainable when only a limited number of profiles with low complexity are to be compared. However, once banding patterns become more complex such as those obtained from intestinal samples or when the number of profiles increases (e.g., in the course of monitoring studies), analysis of SDE fingerprints requires implementation of numerical methods [141]. For this purpose, digitized SDE gels are further processed using dedicated image analysis software like GelCompar and BioNumerics (Applied Maths; http://www.applied-maths.com/), Quantity One and Molecular Analyst (Bio-Rad Laboratories), GeneTools (Syngene; http://www.syngene.com/), and Photo-Capt (Vilber Lourmat; http://www.vilber.com/). These programs permit numerical analysis of band patterns and usually also include statistical approaches for data interpretation. Programs that have been used specifically for statistical analysis of SDE fingerprint data include R (http://www.r-project.org/) and DGGESTAT (developed at the Netherlands Institute for Ecological Research, NIOO-KNAW, Nieuwersluis, The Netherlands).

2.5.1. Diversity and similarity analysis

Most commonly, numerical analysis of SDE profiles relies on the use of diversity indices and/or cluster analysis. Diversity measures for fingerprint analysis such as the Simpson index and the Shannon-Wiener/Weaver index express the degree of ecosystem diversity as a function of band profile complexity but fail to express similarity between profiles based on band positions. Hierarchic clustering algorithms such as unweighted pair-group method using arithmetic averages (UPGMA) produce a visual representation of the similarity between SDE profiles expressed as similarity indices, for example, using the curve-based Pearson product-moment correlation coefficient, the band-based Dice coefficient, or Sorenson's pairwise coefficient. Other authors have used multivariate ordination methods such as nonmetric multidimensional scaling [142, 143], principal component analysis [109, 144], correspondence analysis [145], canonical variate analysis [146], and canonical correspondence analysis [147]. These methods are used for integration of complex datasets such as the bands in an SDE pattern into new mathematical variables which can be projected into a few-dimension perspective or reduced space. A more detailed description of these statistical procedures has been reported elsewhere [148]. Gafan et al. [149] evaluated the use of logistic regression for statistical analysis of complex DGGE profiles. This analysis method takes into consideration the outcome in addition to differences in overall band profile complexity and individual band positions. It is beyond doubt that the list of numerical approaches and statistical tools for analysis of SDE profiles will further expand in the coming years. Although the choice of method(s) is depending on the aim of the study and on the complexity of the ecosystem, community fingerprints generally include more information than are usually revealed with currently available methods. For this reason, more efforts should be put in the development of new and extended processing methods for complex SDE data.

2.5.2. Identification analysis

Next to the first SDE analysis level based on the use of diversity and similarity coefficients, a second level can be defined that allows one to identify and monitor specific members of the intestinal ecosystem. Essentially, identification of individual bands in SDE fingerprints may be obtained by band position analysis (BPA) and/or through band sequencing analysis. Essentially, BPA relies on the comparison of migration distances of band fragments from taxonomically well-characterized reference strains with those of unknown bands present in the sample profiles. BPA can either be performed by analyzing samples and reference strains in adjacent lanes on the same gel (i.e., comigration analysis) or by comparing unknown band positions with those of reference strains present in a user-generated SDE database. In intestinal ecosystems, BPA-based identification may not always yield a conclusive result given the possibility that a single band may consist of multiple amplicons from different species or that two or more (phylogenetically related) species are characterized by the same band position in the sample profile. Ideally, each band position in a sample profile should represent one species. In practice, however, the multioperon effect observed for some taxa when using 16S rRNA gene primers may lead to an overestimation of the number of predominant species in the sample (e.g., see Section 4.2). In contrast to SDE profiles obtained with universal primers, identification of bands in subpopulation profiles by BPA may be more feasible. Application of SDE using group-specific primers for the genera *Bacteroides* [81, 90] and *Bifidobacterium* [74, 90, 108, 117] showed that species identities can be resolved by means of BPA. Temmerman et al. [117] described a protocol to identify bifidobacterial communities based on a nested-PCR-DGGE approach comprising a *Bifidobacterium*-specific PCR step followed by a second PCR step in which both the V3 and V6–V8 regions of the 16S rRNA gene were amplified. A mix of both amplicons was analyzed on a DGGE gel, after which band positions were compared with a user-generated database of reference strains.

Identification results from BPA can or even should be verified by band sequencing, and may help to determine the phylogenetic affiliation of unknown bands. Various procedures have been described to excise and recover PCR fragments from the polyacrylamide gel matrix ranging from conventional elution in electrophoresis buffer to specialized protocols using diffusion buffers and commercial kits [74]. A critical postextraction step during this process concerns the reamplification and subsequent SDE analysis of the excised fragment together with the original environmental sample in order to verify if the correct band was extracted. Upon confirmation, the recovered PCR fragments can be directly sequenced without additional cloning. Subsequent identification of the obtained sequence information can be achieved by comparison with sequences stored in public

databases, for example, EMBL (http://www.ebi.ac.uk/embl/) or GenBank (http://www.ncbi.nlm.nih.gov/genbank).

As further discussed below, the significance of the obtained species information is dependent on the length of the fragment and the hypervariable region it represents in the target gene. This sequence information can also be employed to develop probes for application in FISH and real-time PCR assays to detect and quantify the target organisms. Next to sequencing analysis, identities of individual bands in SDE profiles can also be revealed by Southern hybridization with taxonomic probes [150].

3. ANALYSIS OF HUMAN INTESTINAL MICROBIOTA

The human intestinal tract harbors a highly dense and complex microbial community which plays a pivotal role in maintaining the health status of the gut. Despite the fact that SDE-based methods only allow a superficial view on the microbial diversity and population dynamics of what is considered the predominant part of complex ecosystems, their use in the field of intestinal microbiology has increased exponentially over the past 10 years. The following section aims at reviewing the main contributions of SDE population fingerprinting to our current knowledge on the composition and ecological balance of the human intestinal microbiota linked to health, disease, and dietary intervention.

3.1. Normal intestinal microbiota

Next to a relative minority of organisms belonging to other microbial domains, the human intestinal microbiota mainly consists of bacteria. Although the major site of microbial fermentation is the large intestine (colon), bacterial populations are encountered along the total length of the digestive tract. Starting from the upper bowel, bacterial concentrations gradually increase up to 10^{11}-10^{12}/g in the colon. Parallel to the increase in bacterial density, also the bacterial diversity expands from the small intestine to the colon [151, 152]. From the community point of view, it is important to realize that the intestinal ecosystem evolves from an initially sterile system that becomes successively colonized by various microorganisms.

3.1.1. From newborn to adult

Several studies have used SDE-based techniques to monitor the development of the newborn gut microbiota in humans [6, 96, 101, 102, 106, 107]. At birth, the initially sterile gut becomes inhabited by a variety of bacterial taxa. Succession continues during weaning until a more complex and stable microbiota is established. Two studies by Favier et al. [6, 96] have shown that the intestinal bacterial community of newborns is extremely unstable as evidenced by the fact that many dominant bands in DGGE profiles of fecal samples from healthy full-term babies reduced in intensity, gradually disappeared after a few days and were substituted by other bands. In the first weeks of life, DGGE profiles obtained with universal 16S rRNA gene V6–V8 primers consisted of only a few bands but progressively increased in complexity over time. In combination with clone libraries constructed from 16S rRNA gene sequences, identification of bacterial species corresponding to specific bands in DGGE profiles was possible by BPA. This approach indicated that *E. coli* and *Clostridium* spp. were the main groups among the initial colonizers, which were rapidly replaced by a more complex microbiota consisting of *Bifidobacterium, Clostridium, Enterococcus, Ruminococcus, Enterobacter, Streptococcus, Bacteroides*, and *Actinomyces*. The diversity revealed by DGGE analysis was fairly consistent with previous insights in infant succession patterns based on traditional culture studies [5, 7]. In addition, the successive colonization of the infant gut by bifidobacteria was monitored during the first five months after birth using *Bifidobacterium*-specific primers [96]. Whereas some subjects showed very stable DGGE profiles, others revealed temporal variation in their bifidobacterial population. At each point in time, one to four *Bifidobacterium*-related DGGE bands were observed which always included *Bifidobacterium infantis*. In another study, the dynamics of the developing bacterial community in the neonatal intestinal tract of nine Japanese infants was monitored during the first two months of life [102]. Although the development of individual species was different among the subjects, DGGE profiles of the predominant fecal microbiota together with 16S rRNA gene clone library sequencing revealed a global stepwise evolution from an aerobic to an anaerobic microbial ecosystem. The aerobic organisms that were initially present such as *Pseudomonas* were immediately replaced by facultative anaerobes including *Enterococcus, Streptococcus*, and *Enterobacteriaceae* during the first month. Finally, strictly anaerobic bifidobacteria and clostridia appeared. The establishment and succession of bacterial communities in hospitalized preterm infants tend to follow a different pattern compared to full-term infants [101]. Fecal samples from 29 preterm infants hospitalized in a neonatal intensive care unit and 15 full-term infants were analyzed using DGGE to characterize and compare bacterial succession of the dominant bacterial species in the large intestine. In the first four weeks of life, DGGE patterns increased in complexity over time for all preterm infants. During this observation period, the intraindividual band pattern similarity increased over time as indicated by an increase in Sorenson's pairwise similarity coefficient (C_s) from 0 to 80%. In addition, also the interindividual C_s values increased (18.1 to 57.4%) all of which indicated the acquisition of a highly similar bacterial community in these infants. In contrast, breastfed full-term infants showed a considerably lower interindividual C_s value (11.2%). The strikingly high similarity between bacterial communities from different preterm infants was considered to be associated with hospitalization because the major bacterial groups identified by DGGE BPA belonged to taxa that are routinely isolated in baby care units such as *E. coli, Enterococcus* spp., and *Klebsiella pneumoniae*. This finding thus indicates that the initial colonization of the newborn's intestinal tract is highly dependent on the immediate environment of the individual. In another study assessing the global diversity of the fecal microbiota of preterm infants ($n = 16$), a remarkably low-species diversity and high-interindividual

variability were reported [106]. The low-bacterial diversity was revealed by random sequencing of 16S rRNA gene clones and TTGE analysis. The main fecal groups encountered here included members of the *Enterobacteriaceae* family and of the genera *Enterococcus, Streptococcus*, and *Staphylococcus*. Seven out of 16 preterm infants were colonized by anaerobes, of which four infants were shown to harbor bifidobacteria.

Several studies have documented that bifidobacteria predominate in the fecal flora of breastfed babies, whereas in formulafed infants, other bacterial groups such as coliforms, enterococci, and *Bacteroides* represent the main constituents [7, 153]. In contrast, the possible effect of dietary supplementation in the intestinal development of nursing infants is less well understood. In a longitudinal study, TTGE was used to monitor the predominant and bifidobacterial microbiota of 11 Algerian infants during breastfeeding, breastfeeding with artificial milk supplementation (weaning) and artificial milk alone (postweaning, i.e., cessation of breastfeeding) [107]. In the TTGE profiles, the major bands were assigned by subsequent cloning and sequencing to *E. coli, Ruminococcus* spp., and several *Bifidobacterium* species including *B. longum, B. infantis*, and *B. breve*. Both for the bacterial and bifidobacterial TTGE profiles, distance analysis indicated the expected maturation of the faecal microbiota between 5 and 20 weeks of age, but did not reveal any correlation with the dietary supplementation. Despite a high-interindividual variability, it was observed that the composition of the faecal microbiota appeared more homogenous after weaning which may suggest a correlation with the cessation of breastfeeding. In another study, 65 10-month old infants were included in a randomized dietary intervention study that compared the effect of cow's milk (CM) with infant formula (IF) with or without fish oil (FO) supplement on the diversity of the fecal microbiota [80]. Based on clustering analysis of V3- and V6–V8-16S rDNA DGGE profiling using the Pearson correlation coefficient, it was reported that supplementation of CM or IF appeared to have an influence on the composition of the intestinal microbiota whereas FO intake only showed an effect in the CM group. The authors speculated that these differences may be influenced by the intake of iron and *n*-3 polyunsaturated fatty acids, respectively, but further indepth analysis of the DGGE profiles in combination with other molecular tools is required to substantiate this hypothesis.

Besides the influence of environmental and dietary factors, also the host genotype may have a significant effect on the species composition of the intestinal microbiota. Stewart et al. [84] used TTGE analysis of the predominant bacterial biota to investigate the influence of host genotype on the fecal microbiota in genetically related and unrelated children. In that study, TTGE profiles of identical twin pairs ($n = 13$), fraternal twin pairs ($n = 7$), and unrelated control pairs ($n = 12$) were compared both visually and numerically. Although the community fingerprints of each individual were unique, increased levels of similarity were found between TTGE profiles of genetically related individuals, with the highest similarity values obtained for genetically identical twins (median C_s of 82%) which was significantly different from fraternal twin pairs (median C_s of 68%) and from the unrelated control group (median C_s of 45%). The results of this TTGE study thus suggested that host genetics can have an impact on the composition of the predominant fecal bacterial community in children. Likewise, DGGE analysis of the dominant intestinal microbiota amongst adults displaying varying degrees of genetic relatedness showed that the host genotype had a significant effect on the species composition of the intestinal community [122].

Upon succession, it is thought that a relatively stable intestinal community is established in the adult intestine that appears to be specific for each individual. Zoetendal et al. [95] were the first to report on the stability and uniqueness of the predominant human adult fecal microbiota that can be visualized with SDE-based approaches. TGGE analysis of fecal samples from two healthy individuals showed stable profiles over a period of at least six months which in addition were unique for each individual. These findings were consolidated in a later study [64] in which the host specificity and temporal stability of the DGGE patterns was demonstrated for four subjects over a 16-week period by visual inspection and clustering analysis. In the latter study, also the temporal stability of selected subpopulations was monitored using group-specific primers. DGGE profiles obtained with primers designed to visualize the *Lactobacillus-Leuconostoc-Pediococcus-Weissella*-group tended to show strong temporal variations. Among other autochthonous groups such as the *Bacteroides fragilis* subgroup, however, DGGE profiling using group-specific primers did not reveal such variations. Importantly, the specificity of these group-specific primers was only validated using a set of taxonomic reference strains. A more elaborated strategy was followed in the validation of DNA- and RNA-based DGGE protocols specifically designed to assess the diversity and stability of the *Clostridium coccoides-Eubacterium rectale* (clostridial phylogenetic cluster XIVa) group in fecal samples [70]. In that study, the specificity of the Ccoc-f and Ccoc-r primers was assessed by constructing a clone library in which all 205 DGGE fragments proved to belong to the *Clostridium* cluster XIVa. The authors concluded that the members of this cluster, representing one of the most dominant bacterial groups in the normal intestinal microbiota, followed the same pattern of relative stability as the total predominant population in 12 healthy Finnish adults during six months to two years. Although using protocols differing in sample type, SDE method and primer target, the current view on the uniqueness and temporal stability of the predominant intestinal flora in adult individuals has also been confirmed in other human volunteer studies using SDE-based analyses of fecal samples [77, 81, 83, 98, 99, 118, 154] and mucosa samples originating from different parts of the large intestine [79, 98, 123].

Although the vast majority of SDE-based studies in intestinal microbiology rely on direct DNA extraction from human samples in order to obtain a culture-independent inventory of the microbial diversity, there has also been interest in using DGGE and related fingerprinting techniques to specifically explore the composition of culturable intestinal subpopulations. For instance, DGGE analyses of resuspended bacterial biomass obtained from agar plates of different media selective and nonselective for lactic acid bacteria (LAB) have been used to evaluate the choice of

medium and incubation conditions on LAB recovery and to gain insight in the diversity of culturable fecal LAB in healthy adults [129].

3.1.2. Spatial distribution

The different physicochemical conditions such as pH and concentration of fermentation products prevailing in the ascending, transverse, and descending parts of the colon [155] suggest that also the bacterial composition in each of these three compartments is unique. However, this assumption is not substantiated by SDE-based studies [30, 79, 91, 115, 123]. In most of these studies, DGGE and TTGE fingerprint profiles reflecting the predominant bacterial communities in biopsy samples from different sites of the colon were host specific but highly similar between sites. These findings may indicate that the spatial distribution of at least the predominant mucosa-associated bacterial community is relatively uniform along the length of the colon and its physicochemical gradient. Nielsen et al. [79] reported that DGGE profiles of the bifidobacterial community were relatively simple and consisted of one or two bands for most of the sites sampled along the length of the colon. However, the mucosa-associated subcommunity encompassing the genera *Lactobacillus, Leuconostoc, Weissella, Pediococcus*, and *Aerococcus* produced relatively complex DGGE profiles that varied between hosts and between sampled sites in the colon. In contrast, Zoetendal et al. [123] obtained DGGE profiles with low diversity and little or no variation along the colon when using the same set of group-specific PCR primers. Presumably, the contradictory findings of the two aforementioned studies are due to differences in sampling procedure, DNA extraction method, and/or composition of the subject group.

Given the fact that each individual displays a unique fecal SDE fingerprint [64, 95], investigation into spatial distribution should preferably be based on analysis of a series of site-specific biopsy samples from the same individual. To some extent, this may explain why interindividual comparison of DGGE profiles of single biopsy samples from different sites did not provide any evidence for the existence of site-specific colonization patterns in the human colon [30].

A number of studies have also investigated to what extent the composition of the fecal microbiota reflects the composition of the mucosa-associated colonic microbiota [91, 123]. In these studies, the DGGE/TTGE profiles of amplicons of the variable V6–V8 region of the 16S rRNA gene reflecting the predominant bacterial community of biopsy samples differed significantly from those of fecal samples within the same individual, suggesting that different bacterial populations are dominating the human mucosa and feces. The population diversity revealed by SDE-based community fingerprinting of fecal samples may thus not necessarily reflect the ecosystem composition in other parts of the intestinal tract including the colonic mucosa. This leads to the conclusion that the most accurate information on the diversity and stability of local intestinal communities can thus only be obtained by taking samples through endoscopy or during colonic surgery.

3.2. Intestinal disorders

The pathogenesis of many chronic intestinal disorders and even a number of nonintestinal diseases is believed to be directly or indirectly linked to some members of the indigenous microbiota. Several studies have implemented an SDE-based approach to analyze and monitor the composition and temporal stability of the intestinal microbiota of patients suffering from gut disorders. As an initial approach, SDE techniques permit a rapid and global assessment of microbial diversity without previous knowledge of the composition and are well suited to analyze intestinal microbiota in relation to different experimental conditions and parameters such as healthy versus disease status, active versus quiescent disease phase, different segments of the intestinal tract and response to nutritional or therapeutic interventions. Moreover, the combined use of SDE techniques and quantitative assays such as real-time PCR and FISH that allow to determine the relative concentration of specific indicator organisms offers great potential in this type of studies. The following sections of this review are based on a selected number of studies that have implemented SDE-based methods to assess the potential role of the intestinal microbiota in the (etio)pathogenesis of chronic intestinal disorders.

3.2.1. Inflammatory bowel disease

Although the exact etiology of inflammatory bowel disease (IBD) is not known to date, it is generally assumed to result from an inappropriate response of the mucosal immune system to the normal enteric microbiota in a genetically susceptible individual [156]. It has been hypothesized that specific genetic polymorphisms, such as those in intracellular NOD2 sensors with abnormal function, results in a failure to efficiently regulate expression of Paneth cell-derived antimicrobial peptides [157, 158]. The partial loss of this protective function may allow commensals to damage epithelial cells hereby inducing an inflammatory response. Crohn's disease (CD) and ulcerative colitis (UC) are the two major IBD phenotypes and are characterized by chronic inflammation of the intestinal tract lining which causes severe watery and bloody diarrhoea and abdominal pain [156]. Whereas CD can virtually affect any segment of the intestinal tract, UC is usually confined to the colon and rectum.

The majority of SDE-based studies on IBD have primarily attempted to find differences between CD/UC and healthy fecal or mucosal populations. As such, V3–V5-16S rDNA DGGE profiling and subsequent band sequencing analysis of fresh mucosal biopsy samples revealed a significantly higher prevalence of *Clostridium* spp., *Ruminococcus torques*, and *E. coli* in samples from CD patients (n = 19) compared to healthy specimens (n = 15) [159]. In turn, the butyrate-producing *Faecalibacterium prausnitzii* was more frequently encountered in the latter group. Overall, DGGE fingerprints of mucosal CD populations displayed a higher patient-to-patient variability compared to healthy subjects. The authors postulated that this difference may reflect the difficulty of patients genetically predisposed to CD to maintain and regulate a stable intestinal microbiota.

A study of Bibiloni et al. [160] showed that the phylogenetic composition of biopsy-associated bacteria differed between newly diagnosed untreated CD ($n = 20$) and UC patients ($n = 15$) and healthy subjects ($n = 14$). Biopsies collected from inflamed and noninflamed sites of the terminal ileum and various colonic regions were analyzed by DGGE, 16S rRNA gene clone libraries, and qualitative and quantitative PCR for detection of selected bacterial groups. DGGE profiles of universal V3-16S rRNA gene amplicons were very similar within each subject (mean 85.0 ± 2.4%), irrespective of the intestinal region. However, enumeration by quantitative PCR revealed approximately double numbers of biopsy-associated bacteria for UC patients than CD patients and healthy subjects. In addition, the clone library composition indicated that the composition of biopsy populations in UC and CD patients ($P < .05$), and those from healthy subjects ($P = .05$) were statistically different. This comparison highlighted a significantly higher prevalence of unclassified members of the phylum Bacteroidetes in CD patients, which may indicate that UC and CD are bacteriologically distinct diseases.

Depending on the individual effectiveness, IBD patients undergoing immunomodulatory therapy continuously balance between active disease and remission status. However, it is unclear if and in what way the intestinal microbiota of these patients undergoes compositional changes during these subsequent transitions. In this context, Seksik et al. [24] monitored the fecal microbiota of patients with active colonic CD ($n = 8$), patients in remission ($n = 9$), and healthy volunteers ($n = 16$). TTGE profiles of universal 16S rRNA gene V6–V8 amplicons were very stable over time in the healthy controls but varied markedly for a number of patients ($n = 4$) who were monitored during both active and quiescent phase of CD. Fecal TTGE profiles of these four patients revealed only a slight decrease in the number of bands during the active phase (mean loss of 1.7 ± 2.7 bands), which indicated that the predominant fecal microbiota retained a high degree of diversity in both phases. Based on TTGE band profile composition, no specific bacterial groups could be assigned to active or quiescent CD state. In contrast, quantitative dot blot hybridization of stool samples showed that the fecal microbiota in patients with CD (both active and inactive) differed considerably from those of healthy subjects. Both the *Bacteroides* group (including the genera *Bacteroides, Prevotella*, and *Porphyromonas*) and the bifidobacteria tended to be less represented in CD patients whereas significantly more enterobacteria could be detected. In addition, approximately 30% of the endogenous microbiota of CD patients did not belong to the dominant phylogenetic groups commonly found in healthy controls.

Although currently available data from SDE profiling and other molecular tools implicate a role of intestinal bacteria in CD pathogenesis, a detrimental effect of localized qualitative dysbiosis in CD-associated ulceration has so far not be demonstrated by community fingerprinting. TTGE analysis of biopsy samples of ulcerated and adjacent nonulcerated mucosa of 15 patients with active CD did not reveal qualitative differences in the dominant bacterial population profiles (V6–V8 region of the 16S rRNA gene) within a given patient although a high biodiversity was retained in both cases [92]. Mean similarity values between TTGE profiles of ulcerated and nonulcerated mucosa expressed with the Pearson correlation coefficient did not differ significantly across the different intestinal segments (ileum, right colon, left colon, and rectum) analyzed and ranged from 95.2 ± 4.2% to 97.9 ± 1.7%. Solely based on TTGE analysis using universal primers, it thus appears that local ulceration is not associated with pronounced variation in local bacterial diversity. This conclusion was further substantiated in a later study by the same group on the basis of V3-V4-16S rDNA TTGE profiling and FISH analysis [94]. Also in other studies applying SDE-based population fingerprinting, no particular mucosa-associated microbial pattern could be linked to the (etio)pathogenesis of IBD [91, 123]. Possibly, local dysbiosis among less predominant species may play a role in the pathogenesis of ulceration. Because these minor differences in diversity will largely remain undetected in SDE fingerprinting using universal PCR primers or are difficult to reveal by routine methods for band pattern analysis, future studies should employ group-specific primers to focus on the composition of specific subpopulations and/or should use more indepth mathematical approaches for differential profile analysis.

Whereas most studies concentrated on the inventorization and monitoring of bacterial groups potentially associated with CD, very few studies aimed to address the same question within the metabolically active compartment of the gut microbiota. Sokol et al. [93] analyzed the biodiversity of active bacteria in the dominant fecal microbiota of UC patients ($n = 9$) in comparison with that of healthy subjects ($n = 9$) by applying DNA- and RNA-based TTGE analysis of V6–V8 ribosomal amplicons. The number of bands in DNA-derived TGGE profiles were significantly higher than in RNA-derived profiles for UC patients (15.3 ± 3.2 and 9.1 ± 2.8 bands, resp.) but not for controls (18.3 ± 5.0 and 14.7 ± 5.1 bands, resp.) which indicated a reduction in the biodiversity of the active portion of the fecal microbiota in UC patients relative to healthy controls. Irrespective of the initial template (RNA or DNA), Pearson-UPGMA clustering analysis of TGGE profiles tended to group the samples on the basis of their clinical affiliation (UC versus controls) suggesting that each group has its specific bacterial signature. Interindividual comparison of the "active" microbiota (RNA-derived profiles) revealed a band that was significantly associated with UC patients (89% versus 22% for controls). Sequence analysis attributed this band to *E. coli* or related enterobacteria. Clearly, the possible pathophysiological role of this overrepresentation in the active microbiota of UC patients should be further assessed during remission and within the mucosa-associated microbiota.

3.2.2. Other intestinal disorders

Irritable bowel syndrome (IBS) is an intestinal disorder that is characterized by bowel dysfunction and pain [161, 162]. IBS is a very heterogeneous condition and includes three symptom categories: (i) diarrhoea-dominant, (ii) constipation-dominant, and (iii) alternating type [163, 164]. Although the pathophysiology of IBS is not fully understood,

it is highly probable that alterations in the diversity and stability of intestinal microbiota play a role in the development and/or maintenance of this disorder [165]. In a Finnish study [121], culture-based techniques and DGGE analysis were employed to compare the composition and temporal stability of the fecal microbiota of 21 IBS patients and 17 healthy controls. Culturing revealed slightly higher coliform numbers as well as an increased aerobe/anaerobe ratio in the IBS group. DGGE analysis of 16S rRNA gene V6–V8 amplicons revealed considerable biodiversity and subject specificity of the predominant microbiota in both study groups, but did not identify IBS-specific bacterial groups. Visual comparison of DGGE fingerprints revealed a higher frequency of temporal instability in the predominant bacterial population of IBS subjects (43%) compared to controls (29%). However, profile similarity analysis using the Pearson correlation coefficient revealed comparable interindividual similarity percentages for both groups with a mean similarity of 87.5 ± 11.2% for the IBS group and 85.7 ± 12.7% for the control group. Still, the instability in some of the IBS subjects could partly be explained by disturbances of the intestinal microbiota due to antibiotic therapy during the study. Moreover, the authors suggested that these findings could be associated with a subset of IBS subjects sharing specific symptoms and thus not necessarily reflect the general microbial status of all IBS patients. In this regard, future studies should include subject groups with well-defined symptom-based IBS parameters to evaluate the association of intestinal instability with specific IBS symptoms or with specific bacterial groups and species. In a subsequent study of the same group [71], the predominant and clostridial fecal microbiota of IBS patients and healthy controls were compared to reveal possible differences in the composition, abundance, and stability of selected groups by applying DNA- and RNA-based DGGE analyses and transcript analysis with the aid of affinity capture, a multiplexed and quantitative hybridization-based technique. Clostridia, that is, *C. histolyticum, C. coccoides-Eubacterium rectale, C. lituseburense*, and *C. leptum*, were shown to represent the dominant fecal microbiota in 26 of the 32 subjects under study, contributing altogether 29–87%. The proportion of the *C. coccoides-E. rectale* group was found to be significantly lower in the constipation-type IBS subjects compared to the controls. Although DNA- and RNA-derived predominant community profiles showed considerable biodiversity and subject-specificity, RNA-based DGGE profiles contained significantly fewer amplicons (16 ± 5 compared to 22 ± 5 amplicons). In addition, only RNA-based DGGE profiles of the IBS subjects indicated higher instability of the bacterial population compared to the control subjects. Although intraindividual temporal instability of the predominant microbiota was observed in both IBS and control subjects (with both DNA- and RNA-based DGGE), only RNA-derived DGGE profiles of IBS subjects showed a broader range in similarity values (39–95%) compared to control subjects (68–94%). When considering symptomatic IBS subgroups, the largest intraindividual variability in DGGE similarity values was observed in the diarrhoea-type subgroup. These observations suggest that clostridial microbiota, in addition to the instability of the active predominant fecal bacterial population (RNA-derived profiles), may be involved in IBS. For future research, the use of group-specific primers in SDE analysis focussing on apparently affected groups (e.g., coliforms and clostridia) could be a valuable and effective approach to identify potential IBS indicator organisms.

The use of SDE-based methodologies to determine the diversity and stability of microbiota in inflammatory diseases has meanwhile expanded from IBD and IBS to other intestinal diseases in which dysbiosis of the human microbiome is thought to play a role such as neonatal necrotizing enterocolitis [116] and coeliac disease [130] or diseases beyond the intestinal system such as (atopic) allergies [82, 85, 166] and ankylosing spondylitis [113].

3.3. Intervention studies

Apart from components naturally occurring in a normal diet, also functional foods (including pre- and probiotics) and antimicrobial agents are able to induce beneficial or detrimental changes in intestinal ecosystems. Starting from the first weeks upon birth, the human diet is able to modulate the composition and balance of the intestinal microbiota [7, 153]. The SDE approach is routinely applied in administration studies to monitor the effects on the intestinal microbiota upon consumption of various active components.

3.3.1. Functional foods

The fact that diet is a major factor controlling the human intestinal balance has triggered the development of a new generation of foods specifically designed to strengthen the gut microbiota via modulation. Functional foods include foods and food products with a clearly identifiable health benefit in addition to their basic nutritional value [167]. In functional foods, the addition or incorporation of pro- and/or prebiotic components as active ingredients plays a key role in functional applications aiming at modulation of intestinal microbiota. According to the FAO/WHO definition [168], probiotics are live microorganisms which, when administered in adequate amounts, confer a health benefit for the host. An extended version of this definition is still under debate, including the question whether the live status is truly required for probiotic action [169, 170]. Beneficial effects induced by probiotic activities are mediated either through modulation of the indigenous microbiota or through the immunomodulatory potential of the probiotic strains used. Bacterial cultures incorporated in probiotic products for human consumption commonly—but not exclusively—originate from the intestinal system of healthy (human) subjects and most frequently belong to the bifidobacteria and to LAB such as *Lactobacillus* spp. A prebiotic, on the other hand, is a nondigestible selectively fermented compound that induces specific changes both in the composition and/or the activity of the gastrointestinal microbiota thereby conferring benefits upon host well-being and health [171]. Essentially, the functionality of a prebiotic compound

Table 3: Selection of dietary intervention studies using SDE-based community fingerprinting.

Component[a]	Administered component	Reference(s)[b]
p	Levan-type exopolysaccharides, levan, inulin and FOS	[111]
p	GOS and FOS	[75]
p	Difructose anhydride III (DFA III)	[72, 73]
P	*Lactobacillus rhamnosus* DR20	[76, 88]
P	*Lactobacillus paracasei* F19	[98]
P	VSL#3® (probiotic mixture of eight strains)	[110]
P	*Bifidobacterium longum* (Bifina®) and yogurt with *Bifidobacterium animalis* DN-173 010	[179]
y	*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*	[68]
p, P	Inulin or *B. longum* (Bifina®)	[180]
pP	Inulin-containing probiotic yogurts	[127]
pP	GOS-containing probiotic yogurt	[69]
p, P, pP	GOS and/or *Bifidobacterium lactis* Bb-12	[100]
p, P, pP	Lactulose and/or *Saccharomyces boulardii*	[90]
o	Black tea	[120]
o, op, oP	Isoflavones and FOS or *B. animalis* DN-173 010	[105]

[a] p: prebiotic; P: probiotic; pP: synbiotic; y, yogurt; o: other.
[b] All studies used DGGE as SDE method except in [105], where TTGE was used.

is determined by its potential to stimulate beneficial bacteria indigenous to the gut ecosystem. Complex oligosaccharides are most commonly used as prebiotics including lactulose, galactooligosaccharides (GOS) and fructooligosaccharides (FOS; e.g., oligofructose and inulin). A wide range of beneficial effects have been attributed to probiotics, prebiotics, or a combination thereof (i.e., synbiotics), including modulation of the gut immune system, resistance to microbial infections, antimutagenic/anticarcinogenic effects, reduction of blood ammonia and cholesterol levels, prevention and/or alleviation of diarrhoea and constipation, prevention and reducing symptoms of intestinal chronic disorders, relief of lactose intolerance and increased mineral absorption as reviewed in [172–178].

SDE-based methods have played a key role in human dietary intervention studies aiming at demonstrating the efficacy of functional food components and to substantiate potential health claim. A selection of relevant studies that have contributed to this field is listed in Table 3 [68, 69, 72, 73, 75, 76, 88, 90, 98, 100, 105, 110, 111, 120, 127, 179, 180]. Solely based on findings from SDE analysis, it appears that prebiotic administration can potentially affect the predominant bacterial population of healthy human subjects, whereas most probiotic interventions only seem to induce marked effects in patient groups. This could indicate that some probiotic components may have more of a therapeutic effect in subjects with a disturbed intestinal balance but less effective as general health promoting agents. On the other hand, it should be kept in mind that SDE-based approaches focus on diversity and dynamics of predominant intestinal microbiota, and are as such unsuitable to monitor probiotic interventions that are based on the immunomodulatory potential of the administered organism(s). Bibiloni et al. [110] used DGGE to evaluate the safety and efficacy of the mixed probiotic preparation VSL#3® (http://www.vsl3.com/) consisting of three *Bifidobacterium* strains and five LAB strains (i.e., four *Lactobacillus* strains and one *Streptococcus thermophilus* strain) in patients with active mild to moderate UC. DGGE analysis of V3-16S rRNA gene amplicons generated from biopsies collected from seven patients before and after 6-week VSL#3 administration revealed considerable variation of the predominant microbiota in four out of five patients in remission (mean dice similarity coefficient (D_s) of 69.9 ± 12.7%). In contrast, the DGGE profiles of the two patients with continued active disease remained relatively stable after VSL#3 consumption (mean D_s of 92.3 ± 4.1%). Importantly, it should be noted that the study did not report on the temporal stability of biopsy profiles in the absence of probiotic treatment. In another study, the effect of a 4-week administration of the candidate prebiotic di-D-fructofuranose-1, 2′ : 2, 3′-dianhydride (DFA III) on human fecal microbiota was studied by DGGE analysis using universal V3-16S rRNA primers and *Bacteroides fragilis* subgroup-specific primers [73]. Visual and numerical analysis of the DGGE profiles generated with both primer sets revealed no pronounced changes related to DFA III administration in healthy subjects. In a followup long-term human feeding trial (2 to 12 months) with DFA III, however, DGGE profiles of the predominant bacterial population revealed a marked increase in the intensity of bands related to *Bacteroides* spp. [72]. In a study on the effect of 3-week consumption of a GOS-containing probiotic yogurt on the diversity and temporal stability of fecal microbiota in elderly [69], DGGE revealed that the predominant bacterial population and the *Clostridium coccoides-Eubacterium rectale* group remained relatively stable during the study period. In contrast, the *Lactobacillus* group showed temporal variation which confirms previous observations under basal conditions [64].

In the course of probiotic intervention studies, DGGE and related fingerprinting techniques have been used to verify if the administered strain(s) is (are) detectable in

intestinal samples [76, 88, 98, 100, 105, 110, 125, 179]. Based on a combination of culture-based methods and 16S rDNA DGGE, Wall et al. [86] even reported the recovery of probiotic strains *Lactobacillus paracasei* NFBC 338 and *B. animalis* subsp. *lactis* Bb12 in ileostomy effluents of two infants without a history of probiotic intake. In this context, it should be noted that SDE fingerprinting is not the most optimal tool for detection of administered strains because of the relatively poor detection limit (especially when using universal primers) and the lack of resolution to discriminate the introduced strain(s) from other strains of the same or highly related autochthonous member species of the intestinal microbiota. More suitable approaches are those applying strain-specific primers (e.g., conventional or real-time PCR) or probes (e.g., fluorescent FISH probes) which will not only provide a higher sensitivity but may also allow relative quantification of the probiotic target [181–184]. On the other hand, it should be kept in mind that all aforementioned DNA-based approaches do not allow to discriminate between living and dead cells and thus do not provide information on probiotic survival throughout the gastrointestinal tract.

In recent years, SDE-based community fingerprinting has been integrated in larger polyphasic studies in combination with conventional culture methods and/or with other molecular culture-independent methods to detect and monitor changes in human intestinal ecosystems upon administration of probiotic, prebiotic, or other (in)organic compounds with claimed functionalities. As such, DGGE and FISH approaches were combined with selective culture methods to evaluate the impact of a 3-week diet supplementation with prebiotic GOS or FOS on the composition and activities of the fecal microbiota of 15 healthy human volunteers [75]. V3-16S rRNA gene DGGE profiles remained relatively stable during the study, whereas clear alterations in response to dietary supplementation were observed in rRNA-DGGE profiles as evidenced by the detection of additional fragments or increased staining intensity of band fragments attributed to *Bifidobacterium adolescentis* and/or *Collinsella aerofaciens*. In contrast, DGGE analysis using genus-specific primers derived from the transaldolase gene generated relatively stable profiles for fecal bifidobacteria. Although the taxonomic composition of the bifidobacterial population was not substantially different and both DGGE and FISH revealed that the *Bifidobacterium* and *Collinsella* populations remained relatively unchanged, rRNA-DGGE provided evidence of increased metabolic activity in response to prebiotic consumption. A combination of DGGE and FISH was also used to investigate the effect of black tea drinking on the fecal microbiota of healthy volunteers with hypercholesterolemy [120]. DGGE of 16S rRNA gene V6–V8 amplicons showed that each subject harboured a specific predominant bacterial population that exhibits little change over time and that was not significantly changed by drinking black tea. Even though black tea did not affect the specific bacterial groups analyzed by FISH (i.e., *Bifidobacterium, Bacteroides* and *Prevotella, Clostridium* phylogenetic clustersIV and XIVa, *Atopobium* group, *Faecalibacterium*-like species and *E. coli*), it did decrease the total amount of bacteria detected by the universal bacterial probe. In a study that combined the use of TGGE and FISH analysis, it was demonstrated that isoflavone supplementation with and without pro- or prebiotics induced significant dynamic changes on the composition of the dominant intestinal microbiota of 39 postmenopausal women [105]. Results of FISH analysis indicated that several of the dominant fecal groups were stimulated by isoflavones alone, whereas TGGE profiling of 16S rRNA gene V6–V8 amplicons revealed marked changes in the predominant intestinal microbiota. Intraindividual comparison of TGGE fingerprints showed a mean Pearson similarity value of 73% before and after one month of isoflavone supplementation. In combination with a pro- or prebiotic compound, isoflavones triggered comparable population changes as evidenced by mean fingerprint similarity values of 71 ± 18% and 68 ± 16% obtained for the probiotic (*Bifidobacterium animalis* DN-173 010) and the prebiotic (FOS) test groups, respectively. In addition, FISH results showed a bifidobacterial increase following prebiotic supplementation, often referred to as the bifidogenic effect. Amongst others [76, 125, 180], the aforementioned studies have demonstrated the potential of using SDE fingerprinting and FISH analyses in a complementary approach to characterize basic interactions between intestinal microbiota and functional food compounds and to quantify subpopulations responding to the introduced component(s).

Next to FISH, also real-time PCR has been used in combination with DGGE to verify and substantiate compositional changes in a semiquantitative manner. The latter two methods were used in an integrated approach to monitor and quantify pronounced changes in fecal microbiota of healthy subjects upon long-term administration of a prebiotic (lactulose), a probiotic (*Saccharomyces boulardii*), and their synbiotic combination [90]. Although the DGGE profiles obtained with the universal V3-16S rRNA gene primers as well as those generated using group-specific primers targeting the *Bacteroides fragilis* subgroup, the genus *Bifidobacterium* and the *Clostridium lituseburense* and *Clostridium coccoides-Eubacterium rectale* groups remained fairly stable, one pronounced change was observed in the universal fingerprint profiles after lactulose ingestion. The DGGE band appearing or intensifying in 27 of the 30 subjects could be assigned to *Bifidobacterium adolescentis* by band position analysis and band sequencing. In subsequent real-time PCR analysis, this finding was correlated to a statistically significant stimulation of total bifidobacteria and of *B. adolescentis*. In contrast, the probiotic yeast *S. boulardii* did not display any detectable universal changes in the DGGE profiles nor influenced bifidobacterial levels. In a double-blind crossover study on the qualitative and quantitative effects of fresh and heat-treated yogurt on the bacterial intestinal microbiota from healthy subjects [68], DGGE profiling revealed overall stability of the predominant bacterial population and the LAB population at baseline, after fresh yogurt intake and after heat-treated yogurt intake. However, real-time PCR with group-specific primers indicated a significantly higher density of LAB and *Clostridium perfringens* and a significant decrease in the

density of *Bacteroides* after consumption of both types of yogurt.

3.3.2. Antimicrobial agents

Apart from their generally well-documented therapeutic effects on the site of infection, antimicrobials can also exert a detrimental effect on the microbial balance of the gut ecosystem. So far, studies analyzing the effect of antibiotic therapy on the selection and transmission of antibiotic resistance among pathogens and commensals within the human intestinal microbiota have mainly relied on culture-dependent approaches [185–187] which are highly restricted by the selectivity of the media used. In this respect, SDE-based techniques provide a more suitable approach to monitor the effects of antimicrobial agents on the total community structure of intestinal microbiota.

In several human studies mostly focussing on infant populations, DGGE analysis has revealed drastic alterations among the indigenous bacterial diversity upon therapy with various antimicrobials [77, 96, 101, 102]. Antimicrobial-induced disruptions of fingerprinting profiles were generally accompanied by a reduction in band numbers suggesting an overall decrease in predominant intestinal ecosystem diversity. Favier et al. [96] monitored bacterial succession of the intestine during the first four months of life of five babies, including one infant who received continuous antibiotic therapy consisting of Augmentin (a mixture of clavulanic acid and amoxicillin) for 13 days followed by Bactrimel (a mixture of trimethoprim and sulfamethoxazol) to combat urinary reflux. During the first month after birth, the universal 16S rRNA gene V6–V8 DGGE profiles of the antibiotic-treated baby were highly unstable. Main bacterial groups were identified as *E. coli* and *Enterococcus* spp., despite the fact that the administered antibiotics were expected to suppress enteric bacteria. After one month, DGGE patterns indicated the presence of a simple but remarkably stable community until the end of the study. The most significant differences between the profiles from the antibiotic-treated baby and the other four healthy babies related to the absence of *Bifidobacterium* bands in spite of a partly breast-milk diet. Likewise, a study in which nine Japanese infants were monitored during the first two months after birth demonstrated that antibiotic treatment at the beginning of life exhibits a strong influence on the establishment of a normal microbial ecosystem in the intestine [102]. Two infants who received Cefalex (a cephalosporin antibiotic) therapy in the first four days of life showed a remarkably deviating developmental pattern from the trends observed in the other nontreated subjects. DGGE analysis of V3-16S rRNA gene amplicons generated profiles with an overall low complexity that lacked bands corresponding to bifidobacteria and other strict anaerobes. In fact, band sequence analysis and random sequencing of 16S rRNA gene clone libraries indicated that *Enterobacteriaceae* were the most dominant group throughout the entire study period. In contrast to the findings of the two aforementioned studies, the SDE fingerprinting data reported by Schwiertz et al. [101] indicated that the bacterial composition in infants was not necessarily influenced by antibiotic treatment. In the latter study, the establishment and succession of the neonatal microbiota in the first month of life of 29 preterm hospitalized infants was monitored, and included seven antibiotic-treated infants receiving cefotaxime and piperazine during the first three days followed by vancomycin and amikacin therapy until inflammation was reduced which ranged up to 21 days. Overall, DGGE analysis with universal V6–V8-16S rDNA primers showed relatively stable profiles during and after antibiotic treatment, although the complexity of the banding pattern generally appeared to be lower compared to nontreated infants.

Not suprisingly, it has been shown that the human adult intestinal microbiota is affected to a different degree during antimicrobial therapy depending on the type and/or activity spectrum of the therapeutic component [77]. In the latter study, DGGE analysis of fecal samples from one patient was performed for 12 months during which different antimicrobials were administered. Visual and numerical analysis of the V3-16S rRNA gene fingerprints representing the predominant microbiota remained stable over eight months in the absence of antimicrobials (D_s of 88–91%) and were only minimally affected following one week ingestion of ciprofloxacin (D_s of 73%). In contrast, clindamycin markedly reduced the microbial complexity (D_s of 11–18%). However, once clindamycin therapy ceased, recovery of some intestinal groups was evident within days as indicated by the increasing similarity indices when compared to the pattern prior to antibiotic treatment (D_s of 36–44%). In three other patients, cefazolin (i.e., a cephalosporin with relatively low activity against intestinal anaerobes) caused only minimal alteration of V3-16S rRNA gene patterns (D_s of 81–83%) whereas amoxicillin/clavulanate triggered marked changes in profile compositions (D_s of 19–42%). Overall, the relative degree of alterations in the universal DGGE patterns tended to correspond to the relative activity spectrum of the antimicrobials against intestinal anaerobes.

In order to reduce the possible side-effects of antimicrobial therapy, probiotics are commonly administered in combination with antimicrobials during and after the period of intake [188]. In such combinatorial approaches, the absence of potentially transferable antibiotic resistance genes in the administered strain has been recognized as one of the major safety critera for human probiotics [189]. In this context, the survival and stability of probiotic strains during antimicrobial therapy are particularly relevant but have not been studied into large detail. Upon combined doxycycline (a tetracycline) and probiotic therapy, Saarela et al. [190] found that the complexity of V6–V8-16S rDNA DGGE profiles of fecal microbiota was lower (mean number of bands, 14–25) compared to those of the (control) group only taking probiotics (mean number of bands, 25–42). Probiotic strains *Lactobacillus acidophilus* LaCH-5 and *Bifidobacterium animalis* subsp. *lactis* Bb-12 from the administered commercial preparation Trevis were recovered from fecal samples, and phenotypically and genotypically characterized for their tetracycline (Tc) resistance. The Tc-susceptible strain LaCH-5 remained so during therapy,

whereas recovered isolates of the Tc-resistant strain Bb-12 containing the *tet*(W) resistance gene were not found to have acquired additional Tc resistance genes. Although these observations evidence the stability of the probiotic strains as such, however, the authors did not investigate the possible effect of introducing a *tet*(W)-carrying strain during doxycycline therapy on the dissemination of this gene throughout the intestinal microbiota.

4. LIMITATIONS AND POTENTIAL PITFALLS

Despite its increasing use in the field of molecular microbial ecology, it is clear that SDE-based community profiling has a number of limitations that do not allow indepth analysis of microbial communities as complex as the human intestinal tract. Some of these limitations, such as detection level and taxonomic resolution, can be regarded as potential pitfalls and should be carefully taken into account during protocol development and data analysis. In fact, many of these critical factors are situated along the stages prior to the actual SDE step such as sampling and sample processing, nucleic acid extraction and community PCR, and deserve specific attention when troubleshooting SDE problems.

4.1. PCR bias

As discussed above, the choice of an efficient and reproducible nucleic acid extraction method ensuring optimal cell lysis and maximal removal of various PCR inhibitors present in intestinal samples is highly crucial. Likewise, possible bias introduced during PCR amplification by differential or preferential amplification of target genes from complex communities may prejudice the analysis [191]. As a result, SDE fingerprint profiles may not entirely reflect the actual composition of the predominant microbiota in the sample because of a (partial) lack of amplification of certain DNA/RNA templates. Nonproportional amplification can be due to several factors [192] including template and target sequence properties (e.g., GC-content, presence of secondary structures and template concentration) [191, 193], efficiency of primer binding influenced by primer preference, annealing temperature and primer mismatches, and the number of PCR cycles [104, 194]. Furthermore, it has also been reported that formation of chimeric and heteroduplex molecules during the amplification process [99] may generate a distorted view of the actual microbial diversity [137]. In this context, Petersen and Dahllöf [195] described a new protocol that makes use of internal standards during DNA extraction and PCR-SDE in order to compensate for experimental variability. This modification allows analyzing the relative abundance of individual species back to the original sample, thereby facilitating relative comparative analysis of diversity in complex microbial communities. Other authors have proposed to incorporate an internal standard during PCR to compare fragment staining intensities between profiles and allowing quantitative measurements of fragment intensities [75].

4.2. Taxonomic resolution of the 16S rRNA gene

Although every functional gene can theoretically be used, target genes for SDE fingerprinting should preferably (i) be present in a single copy in the bacterial genome; (ii) contain conserved regions among the members of the population to allow rational primer design; and (iii) comprise regions with sufficient sequence variation amongst the members of the population to produce a fingerprint revealing maximal diversity. Although the 16S rRNA gene is the prototype target in SDE applications based on the above criteria, it should be kept in mind that the possible occurrence of intraspecific multicopy operon heterogeneity [196] and the lack of a sufficient number of polymorphic regions between closely related taxa are intrinsic limitations that may affect the taxonomic resolution and complicate interpretation of SDE fingerprints. Although mostly not recognized as such, both phenomena are sources of systematic error in community fingerprinting analyses [135, 197]. As a result of the multi-operon effect, a single species may appear as several bands instead of a single band in SDE profiles thereby leading to an overestimation of the diversity. For example, Satokari et al. [99] distinguished three distinct DGGE bands when analyzing the amplicon from *Bifidobacterium adolescentis* ATCC 15703^{T} obtained with *Bifidobacterium*-specific PCR primers Bif164-f and Bif662-GC-r (Table 2). Further examination revealed the presence of five rRNA gene clusters in this strain, including two clusters exhibiting microheterogeneity that were visualized as two separate bands. The third visualized band appeared to be a heteroduplex of the former two fragments. Similar observations were detected with the use of other group-specific primers targetting the *Lactobacillus* group [88] and the *Bacteroides fragilis* subgroup [64]. On the other hand, an insufficient number of polymorphic regions in the target gene may lead to an underestimation of the diversity because bands of two or more species have identical positions in the community fingerprint. For example, PCR primers Lac1 and Lac2 specific for the *Lactobacillus* group [88, Table 2] do not allow to distinguish members of the *Lactobacillus casei* group as a result of identical V3-16S rRNA gene sequences. Theoretically, the aforementioned effects can be reduced by choosing the appropriate V region in the 16S rRNA gene [131, 198]. Alternatively, single-copy housekeeping genes characterized by higher substitution rates such as *rpoB* [199–202] have recently been used as targets for microbial community profiling, but still await implementation in intestinal microbiology. To our knowledge, the use of the transaldolase gene in one study for the detection of bifidobacterial populations in fecal samples [74] is the only application in intestinal microbiology using an alternative target gene.

4.3. Taxonomic resolution of SDE profiles

Several authors have identified cases of comigration in SDE analysis of amplicons showing clear sequence variation [203–206]. Even for phylogenetically unrelated strains, it has been reported that the corresponding amplicons might have a similar melting behavior resulting in poor electrophoretic

resolution in SDE [207–209]. The phenomenon of comigration may also cause problems to retrieve reliable sequence information from individual band extracts. To some extent, comigration can be addressed by exploiting a typical advantage of the SDE technology, that is, the use of more narrow gradients in order to produce high-resolution SDE profiles with a particular part of the original profile. This approach has been referred to as denaturing gradient gel electrophoresis gel expansion [210, DGGEGE].

Especially for SDE profiles from complex ecosystems such as the intestinal tract, band sequencing analysis may prove to be less straightforward as anticipated for several reasons. First of all, there is the possibility of multiple sequences being present in a single band due to comigration. In this case, a cloning step should be introduced prior to actual sequencing of the fragments. Furthermore, it has been reported that excised DNA fragments are commonly contaminated with ssDNA originating from other organisms present in the sample resulting in genetic contamination of the sequence profile. Elimination of the ssDNA products through mung bean or S1 nuclease treatment of the eluted DNA prior to amplification (and cloning) can increase the success rate to obtain a pure DNA sequence of the SDE band target [75, 211]. An alternative but more complex approach to overcome both aforementioned problems simultaneously involves direct cloning from the original PCR product followed by screening of individual clones against the environmental sample. In this context, it should be kept in mind that the size range of fragments that can be reliably separated by SDE is limited to 100–600 bp (optimally 200 bp). Sequence analysis of such relatively small fragments may impede reliable identification up to the species level. In addition, in silico and DGGE analysis have revealed cross-reactivity of V3- and V3–V5-16S rDNA primers with the human 18S rRNA gene [119]. Especially in case of biopsies or blood-contaminated fecal samples, coamplification of nontarget eukaryotic DNA with 16S rRNA gene primers may lead to an overestimation of bacterial biodiversity in SDE analysis when no subsequent analysis of individual community amplicons by cloning and sequencing is performed.

Next to their relative electrophoretic position and sequence composition, also the intensity and sharpness of SDE bands require special attention. Artifactual double bands, that is, the situation where each prominent band is accompanied at close distance by a second, often less intense band, have been reported in several SDE applications. Janse et al. [212] suggested that an extension of the final PCR elongation step can be sufficient to prevent the formation of artifactual second bands. The origin of double bands in SDE was explained by the authors as the formation of a secondary product due to prematurely terminated elongation during each PCR cycle. Extended incubation at high temperature during final elongation should disrupt such structures and at the same time allow the *Taq* DNA polymerase to synthesize a complete amplicon. Another observation potentially hampering the resolution of SDE analysis is linked to the phenomenon of extended fuzzy bands. The source of this inconsistency is the existence of multiple melting domains (MMDs) in the amplified fragments which results in a stepwise increase in retardation and ultimately leads to the visualization of a wide and diffuse band. Little is known about the distribution of MMD which is dependent on the target fragment and the phylogenetic group. This phenomenon has been observed when using universal 16S rRNA gene primers in SDE analysis of different types of environmental samples including feces [64], water [204], and soil [213]. Weak, fuzzy bands may erroneously be considered as background smear leading to misinterpretation of the profile richness. Curving down or smiling of bands in lanes near the edges of the gel appears to be an intrinsic feature of any SDE protocol. Although its actual cause is not entirely clear, the smiling effect is thought to result from seeping of urea and/or formamide into the buffer during the run, thereby lowering the concentration of the denaturing substances at the edges of the gel. This effect can be avoided by skipping the outer side lanes during loading and/or by applying silicone grease to the spacers [214].

4.4. Detection limit

The detection limit of SDE-based methods, that is, the minimum (relative) concentration or number in which any given member of a complex bacterial ecosystem needs to be present in order to be visualized in the corresponding community fingerprint, was initially estimated to approach 1% of the total population [49]. This estimation was later substantiated for TGGE analysis of intestinal samples [95], whereas Vanhoutte et al. [64] reported 10^6 CFU/g feces (wet weight) as the detection level that could be reached by DGGE for predominant members of the fecal microbiota. In this context, it should be stressed that the detection limit is a relative value that may strongly depend on several parameters including the taxonomic complexity of the ecosystem present in the sample, the efficiency of DNA extraction, the total number of bacteria, and the relative concentration of each organism in the sample. Human stool usually contains 10^{10}–10^{12} CFU/g feces, and it is thus possible that the detection limit may improve if 10^{10} CFU/g compared to 10^{12} CFU/g is present due to a lower competition among the constituting DNA templates during PCR amplification. In general, the potential to detect a specific taxon can be improved by using group-specific primers that can narrow the size of the target population. Even when using a genus-specific primer, however, the template DNA ratio may still affect the DGGE-based detection of certain species that are underrepresented in a mixed community sample [128]. Although poorly studied for SDE-based community fingerprinting of human microbiota, multiple displacement amplification (MDA) may provide another strategy to enhance the detection level especially in biopsy samples with lower bacterial counts. In MDA, the use of 3′ to 5′ exonuclease resistant random oligonucleotide primers and bacteriophage Phi29 DNA polymerase will enrich any DNA target [215], and the resulting template pool can be used for 16S rDNA PCR and subsequent SDE profiling, for example, using TTGE [216].

5. CONCLUSIONS AND FUTURE PERSPECTIVES

This review has highlighted the broad application spectrum of SDE-based techniques in the field of intestinal microbiology, ranging from primary assessments of the bacterial complexity and diversity of intestinal community structures to the monitoring of compositional changes at different population levels upon dietary or therapeutic interventions. In more advanced approaches, additional tools such as band sequence analysis, band position analysis, and blotting analysis permit further taxonomic exploration of the microbial communities present in the gut. Overall, SDE techniques are technically fairly simple, fast, flexible, and reproducible. Because they allow simultaneous analysis of multiple samples, SDE-based methods may be highly suitable in the selection of candidate subjects for human metagenome studies. Taken together with the ability to visualize poorly or as yet unculturable bacterial groups, these features have contributed to the current fame and reputation of SDE technology.

As is the case with any other methodology, however, also the SDE approach has a number of intrinsic limitations. Besides the general biases associated with sampling (including sample size), total DNA extraction and PCR amplification, also more specific restrictions such as intraspecies 16S rRNA gene operon heterogeneities, limited fragment length, or fuzzy bands can limit the applicability of SDE. On the other hand, it is important to always consider the significance and possible consequences of these drawbacks in the context of the study because some limitations will not be equally important when monitoring community stability compared to when assessing biodiversity. One of the most important steps in the definition of a new SDE protocol is the choice of the primer target which can already prevent several potential drawbacks related with SDE. A careful selection of the target fragment with regard to sequence variability and the distribution of multiple melting domains and a clear focus on the phylum of interest is conducive to achieve the desired resolution. In addition, also the SDE technology itself is constantly developing. Denaturing high-performance liquid chromatography (DHPLC) has relatively recently been introduced to detect genetic variation based on the SDE principle, but employs an HPLC column instead of a polyacrylamide gel matrix for amplicon separation [217]. When integrated in fully automated instruments such as the Transgenomic WAVE systems (http://www.transgenomic.com/), DHPLC analysis offers several advantages over conventional SDE analysis including the lack of gel preparation, the higher throughput, and the possibility to automatically collect sample fractions for further (sequence) analysis. DHPLC has been successfully used to analyze microbial communities with a low [218, 219] or high [220] complexity. One particular study in the field of intestinal microbiology revealed that DHPLC provides a number of technical benefits compared to DGGE but appears to have the same limitations in taxonomic resolution for profiling 16S rRNA gene amplicons [97]. Other emerging technologies such as the combination of isotopically labeled substrate analysis with RNA-DGGE [221] may offer a promising prospect for implementation in functional studies on gut microbiota.

In contemporary intestinal microbiology, SDE-based methods are rarely used as a single or end-point approach but are usually combined with culture methods and/or other molecular methods such as clone libraries, FISH, real-time PCR, and microarrays in a complementary research strategy. It is beyond doubt that these polyphasic study designs should be further pursued and developed to broaden current insights in the microbial diversity, dynamics, and interactions within the intestinal tract. In this regard, one of the major challenges ahead lies in the combined analysis of microbial presence and microbial activity. As an example of such an integrated approach, parallel DGGE analysis targetting the 16S rRNA gene as taxonomic marker and the adenosine-5′-phosphosulfate reductase subunit A gene as functional gene has been used to study the succession and diversity of sulfate-reducing bacteria in the mouse gastrointestinal tract [222]. In this regard, the wealth of information expected from large-scale sequencing efforts such as the Human Microbiome Project (http://www.nihroadmap.nih.gov/hmp/) may open new avenues for the development of SDE primers targeting specific functional genes. Considering all upcoming technological developments, it is expected that SDE community profiling will maintain and even reinforce its position in the large spectrum of molecular approaches currently employed to unravel host-microbe and micromicrobe interactions within the human microbiome. The successful incorporation of DGGE profiling in the recently launched concept of functional metagenomics [223], that is, the transgenomic characterization of key functional members of the microbiome that most influence host metabolism and hence health, brings forward a first line of evidence in that respect.

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Research Article

Ecological Characterization of the Colonic Microbiota of Normal and Diarrheic Dogs

Julia A. Bell,[1,2] Jamie J. Kopper,[1,2,3] Judy A. Turnbull,[1,2] Nicholas I. Barbu,[1,2] Alice J. Murphy,[1,2] and Linda S. Mansfield[1,2,4]

[1] *Comparative Enteric Diseases Laboratory, National Food Safety and Toxicology Center, Michigan State University, 181 Food Safety Building, East Lansing, MI 48824, USA*
[2] *College of Veterinary Medicine, Michigan State University, G-100 Veterinary Medical Center, East Lansing, MI 48824, USA*
[3] *Cell and Molecular Biology Program, Michigan State University, 2240A Biomedical and Physical Science Building, East Lansing, MI 48824, USA*
[4] *Department of Microbiology and Molecular Genetics, Michigan State University, 2215 Biomedical Physical Sciences, East Lansing, MI 48824, USA*

Correspondence should be addressed to Linda S. Mansfield, mansfie4@cvm.msu.edu

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We used terminal restriction fragment polymorphism (T-RFLP) analysis to assess (1) stability of the fecal microbiota in dogs living in environments characterized by varying degrees of exposure to factors that might alter the microbiota and (2) changes in the microbiota associated with acute episodes of diarrhea. Results showed that the healthy canine GI tract harbors potential enteric pathogens. Dogs living in an environment providing minimal exposure to factors that might alter the microbiota had similar microbiotas; the microbiotas of dogs kept in more variable environments were more variable. Substantial changes in the microbiota occurred during diarrheic episodes, including increased levels of *Clostridium perfringens, Enterococcus faecalis*, and *Enterococcus faecium*. When diet and medications of a dog having a previously stable microbiota were changed repeatedly, the microbiota also changed repeatedly. Temporal trend analysis showed directional changes in the microbiota after perturbation, a return to the starting condition, and then fluctuating changes over time.

1. INTRODUCTION

Acute infectious diarrhea is a worldwide public health problem with a long list of differential diagnoses. In the US, major pathogens responsible for most cases include *Salmonella, Campylobacter, Shigella, Escherichia coli* O157:H7, and *Cryptosporidium* [1]. *Vibrio, Yersinia, Listeria, Cyclospora, Clostridium difficile, Giardia*, rotavirus, and *Entamoeba histolytica* are also reported at lower rates [1]. Enterotoxigenic, enteropathogenic, enteroaggregative, and enteroinvasive strains of *E. coli,* toxin-producing *Clostridium perfringens, Staphylococcus aureus, Bacillus cereus,* and norovirus also cause infectious diarrheas, but may not be included in routine testing [1]. Main etiologies for acute diarrhea in the dog are the same or similar to organisms seen in humans [2–6]. Other known pathogens of dogs include *Clostridium piliforme* [7], *Brachyspira* (*Serpulina*) spp. [8], *Enterococcus* spp. [9], and *Helicobacter* spp. [10–12]. In both humans and dogs, a number of bacteria, such as *Enterococcus* spp. and certain *Clostridia* spp., are recognized as opportunistic pathogens or enhance disease from other organisms when conditions are ideal for their growth and when competitors are absent [13–15]. Most cases of acute infectious diarrhea are self-limiting illnesses and resolve in a few days with or without symptomatic treatment with rehydration along with antimicrobial or antiparasitic drugs targeting the "etiological agent" [16]. The presence of known GI tract pathogens recovered or demonstrated is used to attribute an etiology during a diarrheic episode; however, causation is seldom proved.

Few animal models of functional GI disorders exist, but the dog GI tract and microbiota bear many similarities to those of humans. Dogs are monogastric omnivores in which dietary manipulations are easy to achieve and a small-sized

cecum provides only a small component of hind gut fermentation. Dogs are litter bearers with a short reproductive interval, where families of related individuals are easy to acquire and where management can be manipulated to control environmental exposures. Early work has been done to describe GI microbiota in healthy dogs. Microbial community analysis of feces from 4 Labrador breed dogs was performed using culture followed by 16S rRNA gene sequencing [17]. Despite intensive efforts, these methods underestimated community diversity and skewed results toward organisms more successful on particular culture media. These results did support the use of molecular-based methodologies for determining community profiles, but at the time, sequences of many isolates were not found in the Ribosomal Database Project and EMBL databases. Suchodolski et al. described the microbial community in duodenum, jejunum, ileum, and colon contents from six healthy unrelated dogs using near-full-length 16S rRNA gene PCR and cloned libraries [18]. Here, *Firmicutes* was the most diverse and abundant phylum; *Clostridiales* was the most diverse bacterial order, forming several *Clostridium* clusters; anaerobic *Fusobacteriales* and *Bacteroidales* increased in their relative abundance along the intestinal tract, peaking in ileum and colon; and *Lactobacillales* occurred commonly in all parts of the intestine. These results on *Firmicutes* and *Clostridiales* were similar in humans [19] with *Clostridium* cluster XIVa being the predominant contributor to *Clostridiales* sequences in both dogs and humans. Furthermore, *Fusobacteria* appeared to be a minor part of the intestinal community in other species, including humans [19]. Also, *Proteobacteria*—including *E. coli*-like organisms—predominated in the duodenum and were sparse in the colon in both dogs [18] and humans [19]. In another study designed to define host distribution patterns of fecal bacteria of the order *Bacteroidales* as markers for fecal source identification in aquatic environments, human, dog, cat, and gull sequences were clustered together in phylogenetic analysis [20]. Swanson et al. performed a study using healthy dogs to examine whether prebiotic (fructooligosaccharides) or probiotic (*Lactobacillus acidophilus*) treatments would alter gut microbial populations, fermentative end products, and nutrient digestibilities [21]. In one experiment, fructooligosaccharide treatment decreased *C. perfringens* and increased fecal butyrate and lactate concentrations, while in a second experiment, this treatment increased bifidobacteria, lactobacilli, and fecal lactate and butyrate and decreased fecal ammonia, isobutyrate, isovalerate, and total branched-chain fatty acid concentrations. Finally, recent global vertebrate gut microbiota studies showed that captive (zoo) bush dogs on carnivorous diets had microbial communities that clustered with other carnivores, and that primates on omnivorous diets had fecal microbiota most like humans [22, 23]. In these studies using tree-based and network-based analyses of microbial communities, clustering by diet (herbivore, omnivore, or carnivore) was highly significant. However, these investigators did not consider the microbiota of modern pet dogs on highly processed diets and cohabiting with humans. Taken together, these studies show that dogs are a reasonable model for study of the role of microbiota in GI disorders and that understanding of the GI microbial community in dogs is at a stage of readiness for this to be pursued.

Many diarrheal diseases are attributable to specific pathogens, to polymicrobial interactions, or to shifts or imbalances in the resident microbial community in response to external stress(s). Thus, acute diarrheas can result from myriad etiologies making attribution difficult. Lately, attention has been focused on the role of the microbiota. The normal gut biota or "enterome" is a complex microbial ecosystem that plays a crucial role in maintaining GI homeostasis and in certain disease states [24]. However, 300–500 different bacterial species are estimated to inhabit the human colon, many of which are not cultivatable [25]. This estimate of diversity has changed little over the years, even with the application of molecular techniques to the study of the colonic microbiota [26, 27]. Eckburg et al. [26] used collector's curves to estimate that extensive sequencing would reveal at least 500 species. Adding to complexity during analysis are the sheer numbers of GI organisms, which can reach a density of 10^{12} organisms/gram of feces, with a total gut population of 10^{14}–10^{15} microbes [28]. In the dog, breed and age were shown to have significant effects on particular aerobic and anaerobic bacterial counts using denaturing gradient gel electrophoresis of PCR amplified 16S ribosomal fragments. Here, each individual dog harbored a characteristic fecal bacterial community which was independent of diet [29]. We hypothesized that dogs have a stable composition of the colon microbial community and that episodes of diarrhea lead to long lasting changes in community composition and/or function; furthermore, treatment for specific pathogens can compound these effects. To address this hypothesis, we required (1) diarrheic perturbations of the GI tract with or without treatment to study, (2) documentation of the presence of pathogens in the GI tract, (3) a cost-effective technique for assessing shifts in the GI tract microbiota, and (4) the assurance that the microbiota in an individual is stable enough for us to be able to detect meaningful changes. We used diagnostic PCR assays to document the presence of pathogens in the GI tract. The GI tract perturbations we studied were (1) acute episodes of diarrhea with or without antibiotic treatment and (2) changes in diet and medications. The technique we chose for assessing shifts in the GI tract microbiota was terminal restriction fragment length polymorphism (T-RFLP) analysis.

T-RFLP is one of a family of related techniques used to describe microbial communities containing large numbers of organisms that are undescribed and/or difficult to cultivate. T-RFLP is based on PCR amplification and restriction enzyme digestion of 16S rDNA PCR products followed by capillary electrophoresis on a DNA sequencer. Here, data were analyzed using exploratory statistical techniques that help reveal patterns rather than the more familiar inferential statistics that help discriminate between hypotheses. Community studies using these techniques that have been reported to date have involved small numbers of samples. For example, Nielsen et al. characterized populations of

Table 1: Characteristics of dogs enrolled in the study.

	Breed	Sex	Age	Diet (%)			Comments
				protein	fat	fiber	
Pet 1	Beagle × schnauzer; related to research colony dogs	F	4 years	21.5	13.0	3.0	Time series; one diarrheic samples (dietary indiscretion; no treatment), three normal samples
Pet 2	Labrador retriever puppy; same household as Pet 1	M	9 weeks	26.0	8.5	3.0	Time series; six normal samples
Pet 3	Golden retriever	M	12 years	25.0	6.0–8.0	12.0	Time series with normal and diarrheic samples (dietary indiscretion; metronidazole treatment)
Pet 4	Golden Retriever	M	6 years	22.0	12.0	5.0	One normal sample
Pet 5	Dachshund	M	7 years	22.0	13.0	3.0	Normal sample
Pet 6	American Eskimo mix	M	3 years	22.0	13.0	3.0	Normal sample
Pet 7	Retriever mix	F	2 years	ND	ND	ND	Diarrheic sample
Pet 8	Mixed breed	F	12 years	ND	ND	ND	Diarrheic sample
Research 1	Beagle × Schnauzer	F	4 years	25.0	9.0	5.0	Normal sample
Research 2	Beagle × Schnauzer	F	6 years	25.0	9.0	5.0	Normal sample
Research 3	Beagle × Schnauzer	M	4 years	25.0	9.0	5.0	Normal sample
Research 4	Beagle × Schnauzer	F	6 years	25.0	9.0	5.0	Normal sample
Research 5	Beagle × Schnauzer	F	4 years	25.0	9.0	5.0	Normal sample

lactic acid bacteria and total bacterial communities in one sample each from three colon segments in four human subjects [30]. In this study, the total communities from different parts of the colon in the same individual were similar to each other, but total communities varied between individuals. In another trial, fecal communities were studied in eight individuals of different ages and sexes; five adults and two children had similar arrays of microorganisms in their fecal communities but different proportions of the bacterial species, while a two-week-old infant had a much simpler community [31]. Thus, T-RFLP data revealed valuable results even though attribution of gains or losses of specific bacterial genera or species was not possible. Also, we recognize that many factors may affect the results obtained with the T-RFLP technique; these factors include choice of primers; choice of restriction enzymes; and various amplification biases due to PCR reaction parameters such as amount and complexity of the template DNA, annealing temperature, and number of cycles in the amplification reaction [32–35]. Nevertheless, T-RFLP is rapid, sensitive, and reproducible [31, 34], and, unlike many other community analysis techniques, it yields both taxonomic information regarding organisms in the community and estimates of their relative proportions in the total microbial population. It is considered a useful tool in the study of microbial communities and can be used to generate data that help to determine whether further studies employing more precise, laborious, and expensive techniques, such as targeted real-time PCR for the detection and quantization of specific microorganisms or the generation, sequencing, and analysis of cloned 16S rDNA libraries, are justified [31].

To address our hypothesis, we studied the microbial communities of dogs during diarrheic episodes and compared them to those of healthy control dogs to make a preliminary assessment of the contribution of members of the normal community to acute diarrheal disease processes. Results of these studies showed that fecal microbiotas varied among dogs, even those that were closely related, and were largely influenced by diet. A dog treated for diarrhea with metronidazole did exhibit loss of richness followed by return to a stable microbiota; the same treatment aimed at a second bout of diarrhea resulted in an unstable microbiota that ultimately lost richness and evenness. Thus, our hypothesis, that dogs have relatively stable colon microbial communities and episodes of diarrhea lead to instability which is compounded by antimicrobial treatments for specific pathogens, can be addressed using these methods particularly if environmental exposures are limited. This work demonstrates that dogs can be used to study changes in microbial communities associated with naturally occurring diarrheas.

2. MATERIALS AND METHODS

2.1. Enrollment of study dogs and experimental design

We established a standard operating procedure for sample collection and processing that was approved by the Michigan State University Institutional Animal Care and Use Committee (2-16-2006). The study also followed all guidelines and standard protocols of the Michigan State University (MSU) Veterinary Teaching Hospital (VTH). Information on all animals included in the study is given in Table 1. A panel of eight household pet dogs was enrolled in the study

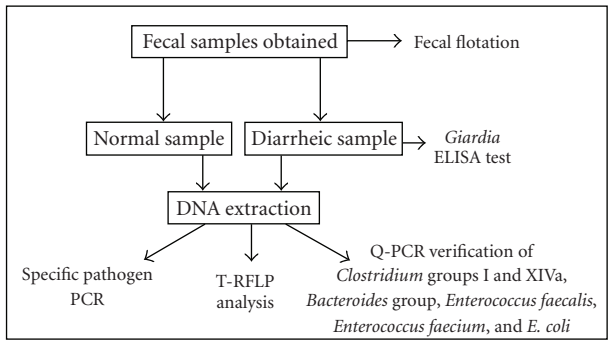

Figure 1: Scheme for sample processing.

over one year. These eight dogs included two pets from a single household (Pets 1 and 2 in Table 1; Pet 1 suffered one episode of diarrhea), a single pet from a second household (Pet 3 in Table 1; this pet suffered two episodes of diarrhea which were treated at the MSU VTH), a single healthy pet from a third household (Pet 4 in Table 1), two healthy pets from a fourth household (Pets 5 and 6 in Table 1), and two pets from different households presenting to the MSU VTH with diarrhea (Pets 7 and 8 in Table 1). Five control dogs from an extended genetically related family were enrolled that were housed in an MSU-closed research colony, were fed the same food daily, and were exercised indoors to prevent infections. Feces were collected from these dogs to assess repeatability and technical quality of our protocols and to provide comparisons to diarrhea cases.

All diagnostic tests (except for an ELISA test for *Giardia*) and T-RFLP analyses were conducted on DNA isolated from fecal samples. DNA of sufficient quality for T-RFLP analysis could not be isolated from one of two samples from Pet 4 or from Pets 5, 6, 7, or 8. Diagnostic PCR results are presented for all thirteen dogs: T-RFLP results are presented for the five research colony dogs and four household pet dogs (Pets 1, 2, 3, and 4). For T-RFLP analysis of fecal microbiota over time, we collected repeated fecal samples from three household pet dogs to assess variability of the microbiota based on housing and feeding regimes. As mentioned above, two of these three dogs experienced diarrhea, one of them twice. Therefore, we collected samples from these dogs during and after treatment, if any, with the prescribed antibiotic.

2.2. History and clinical examination

The overall strategy for sampling of dogs is shown in Figure 1. The case definition for a dog with diarrhea was a dog that presented with acute diarrhea. If dogs presented to the MSU VTH with diarrhea, we instituted resampling three to five days after completion of treatment. For one of the two diarrheic household pets studied (Pet 3 in Table 1), two original samples were taken before antibiotic treatment and further samples were taken during and after antibiotic treatment; for Pet 1 in Table 1, the initial sample was diarrheic and subsequent samples were taken after resolution of disease, which was not treated.

With cases presenting with signs of acute large bowel diarrhea, the typical MSU small animal clinic protocol includes a fecal flotation, *Giardia* test, and fecal cytology. Treatment usually comprises a short course of metronidazole (10–50 mg/kg BID) and institution of a low-residue diet. In this study, our protocol involved taking a history, performing a physical examination, and initiating diagnostic tests according to standard methods currently employed in the MSU VTH.

2.3. Sample handling and DNA isolation

Preliminary studies were conducted to define the best method of handling fecal samples to optimize T-RFLP analysis. Using clean gloves for each animal, samples from research colony dogs and Pets 1 and 2 were taken as free catch or rectal samples; samples from other dogs were taken from the ground immediately after defecation taking care to avoid taking any part of the sample that touched the ground. Subsamples were taken from the interior of the fecal mass for analysis. The feces was placed into tryptose soy broth with 15% glycerol, mixed well, aliquoted into at least 4-5 identical subsamples; three were frozen back at −80 and one had DNA extracted that day. Samples were subjected to one of the following treatments: holding on ice only long enough for transport to the laboratory, holding at room temperature for 24 hours prior to DNA extraction, holding on ice for 24 hours prior to DNA extraction, refrigeration for 24 hours prior to DNA extraction, and freezing for 24 hours prior to DNA extraction. These conditions were intended to mimic the possible fates of clinical specimens prior to submission to the laboratory. DNA could be recovered in quantities sufficient for diagnostic PCR and T-RFLP analysis from samples subjected to all treatments, although yields were greater when samples were held on ice only long enough for transport to the laboratory. Freezing was preferred when samples could not be processed immediately in order to avoid changes in microbiota that have been documented in samples held at room or refrigeration temperatures [36–38].

Bacterial populations were recovered from 200 mg of feces by suspending samples in 300 mM sucrose solution followed by two low-speed centrifugations as described [39]. Bacteria were then collected by high-speed centrifugation and the pellets resuspended in 200 microliters 10 mM Tris 1 mM EDTA, pH 8.0. Klijn et al. [39] reported that this method results in the recovery of over 80% of aerobic/facultatively anaerobic bacteria able to grow on Columbia blood agar medium (they did not assay obligately anaerobic bacteria). Similar differential centrifugation methods have been used to isolate bacterial DNA for microbial community analysis from digesta from chicken gastrointestinal tracts [40, 41], rat cecal digesta [42], and human feces [43]. Community DNA was isolated from the harvested bacterial cells using QIAgen DNeasy Tissue Kit (QIAgen, Valencia, Calif, USA) according to the manufacturer's instructions for Gram-positive bacteria, including digestion first with 20 mg/mL lysozyme for one hour at 37°C followed by proteinase K (20 mg/mL) overnight at 55°C;

DNA was then purified from the lysates using QIAgen spin columns.

2.4. Screening for common pathogens in normal and diarrheic dogs

Fecal samples were screened for parasites by the Cornell-Wisconsin saturated sucrose flotation technique [44]. *Giardia* tests were performed using the ProSpect Giardia-ELISA-microplate assay (Remel, Lenexa, Kan, USA) according to the manufacturer's instructions. DNA preparations were screened for the presence of DNA from the following bacterial pathogens by standard polymerase chain reaction assays using published primer pairs and cycling conditions: *Clostridium perfringens* [45], *Campylobacter* spp. [5], *Enterococcus* spp. [46], *Enterococcus faecium* [47], *Helicobacter* spp. [48], *Salmonella* spp. [49], and *Brachyspira (Serpulina)* spp. [50]. Purified DNA samples from cultured known bacterial species were used as positive controls for the PCR assays. In addition, quantitative PCR assays (Q-PCR) were performed using primers and cycling conditions developed by Rinttilä et al. [51] for *Bacteroides* spp. and related organisms, *Clostridium* group I, and *Clostridium* group XIVa. However, Q-PCR assays for *Escherichia coli* were performed as published [52]; Q-PCR assays for *Enterococcus faecium*, and *Enterococcus faecalis* were performed as described below [53].

2.5. T-RFLP analysis

Terminal restriction fragment polymorphism analysis was conducted using the 516f and 1510r primers, PCR reaction mixture, PCR cycling conditions, and restriction enzyme digestion conditions described by Nagashima et al. [31], except that reactions were carried out using 50 ng template DNA in a total volume of 100 μL. The forward primer carried a 6-FAM fluorescent probe. The PCR products were purified using QIAquick PCR purification columns (QIAgen; Valencia, Calif, USA) according to the manufacture's protocol prior to digestion with *Bsl*I (New England Biolabs, Inc., Ipswich, Mass, USA); the resulting fragments were separated by electrophoresis on an automated DNA sequencer (ABI Prism 3100) at the MSU Genomic Technology Support Facility. An internal lane standard (MapMarker1000; BioVentures, Murfreesboro, Tenn, USA) was added to every sample, and the standard peak sizes were used by the GeneScan Analysis software to compute peak sizes. Electropherograms were stored as computer files for later analysis.

2.6. T-RFLP data analysis

Analysis of T-RFLP *Bsl*I peak patterns was conducted as follows. Only peaks corresponding to DNA fragment lengths between 100 and 990 bp in length, having a height of at least 25 fluorescent units, and contributing at least 1% of the total area under the electropherogram were considered; electropherograms having a total area less than 5000 fluorescence units were not analyzed. Likely identities of the phylogenetic groups of bacteria detected were determined manually by comparing peak fragment sizes to fragment sizes assigned to various bacterial groups by Nagashima et al. [31], allowing for an error of ± 1 bp. Peaks that did not fall into the size classes defined by Nagashima et al. [31] were combined to form an "unknown" class. The identities of the bacterial groups and the range of peak sizes that contributed to each group are shown in Table 2. The community profile consisted of a list of the bacterial groups present or absent in a sample and the % area under the electropherogram contributed by each group.

For cluster analysis, the % area data were fourth-root transformed, and single linkage cluster analysis using the Bray-Curtis similarity index was performed on the transformed data [54]; a dendrogram was constructed, and its stability was evaluated using the jackknife procedure [55]. These calculations were performed using utilities made available online by John Brzustowski at http://www2.biology.ualberta.ca/jbrzusto/cluster.php. The Bray-Curtis similarity index is widely used in community ecology studies because it is less affected than other indices by differences involving rare, low abundance organisms and it is thought to perform better with datasets containing widely differing sets of communities [55, 56]. This index takes into account both the presence and absence of peaks and differences in the areas under same-sized peaks in the electropherograms, which indicate differences in the proportions of a particular organism in two populations.

We also calculated the descriptive microbial community parameters developed specifically for molecular ecological fingerprinting by Marzorati et al. [57]; the community parameters summarize community richness (R_0), functional organization (*Fo*, evenness), and dynamics (% change and Δ_t). These parameters were calculated and interpreted for T-RFLP profiles generated in this study as described in Marzorati et al. [57], except that the Bray-Curtis similarity index was used in the calculation of the % change parameter instead of the similarity index used by Marzorati et al. [57]. Finally, patterns of variability in T-RFLP profiles for Pet 3 over time were analyzed by the regression method of Collins et al. [58]. Euclidean distances between microbial communities at different time points were calculated using utilities made available online by John Brzustowski at http://www2.biology.ualberta.ca/jbrzusto/cluster.php; regression analysis was performed using SigmaStat 3.1 (Systat Software, Point Richmond, Calif, USA).

2.7. Quantitative real-time PCR method and analyses

DNA extracted from feces (as previously described) was used as the template in species-specific Q-PCR assays. *Bacteroides/Prevotella/Porphyromonas* and *Clostridium* groups I and XIVa assays were performed using the primer sequences from Rinttilä et al. [51]. *Escherichia coli* Q-PCR assays were performed using primer sequences from Khan et al. [52]; *Enterococcus faecalis* and *Enterococcus faecium* Q-PCR assays were performed using primer sequences from Firmesse et al. [53]. In all assays, 25 μL reactions were performed in triplicate for each sample with iQ SYBR Green Supermix (Bio-Rad, Hercules, Calif, USA) and 250 ng of fecal DNA.

Table 2: Assignment of T-RFLP fragment size classes to bacterial taxa based on data of Nagashima et al. [31].

Fragment class according to Nagashima et al. [31]	Fragment size range (bp)	Predominant genus	Other genera included
*Bsl*I 110	110–115	None	*Clostridium, Eubacterium, Lactobacillus, Veillonella*
*Bsl*I 124	125–128	*Bifidobacterium*	None
*Bsl*I 317	316–319	*Prevotella*	*Lactobacillus*
*Bsl*I 332	326–338	*Streptococcus*	*Bifidobacterium, Clostridium, Eubacterium, Lactobacillus, Prevotella*
*Bsl*I 370	364–378	*Clostridium*	*Bacteroides, Bifidobacterium, Eubacterium, Lactobacillus, Prevotella*
*Bsl*I 469	464–473	*Bacteroides*	*Clostridium, Eubacterium, Prevotella*
*Bsl*I 494	487–502	*Clostridium*	*Eubacterium, Ruminococcus, Streptococcus*
*Bsl*I 520	513–519	*Enterococcus*	*Clostridium, Eubacterium, Lactobacillus*
*Bsl*I 657	655–665	*Lactobacillus; Streptococcus*	*Bacteroides, Clostridium, Enterococcus, Eubacterium, Ruminococcus*
*Bsl*I 749	748–757	*Clostridium*	*Eubacterium, Fusobacterium, Ruminococcus*
*Bsl*I 853	848–854	*Bacteroides*	*Bacteroides*
*Bsl*I 919	911–921	*Ruminococcus*	*Enterococcus, Eubacterium*
*Bsl*I 940	935–941	*Escherichia*	*Clostridium, Eubacterium, Fusobacterium, Ruminococcus*
*Bsl*I 955	955–960	None	*Clostridium, Eubacterium, Ruminococcus*

The following cycling protocol was used for *Clostridium* group I, *Clostridium* group XIVa, *Bacteroides*, and *E. coli*: 95°C for 3 minutes and 40 repeats of 95°C for 10 seconds, specific annealing temperature for 30 seconds. Cycling parameters for *E. faecium* were 95°C, 3 minutes; 40 cycles of 63°C for 10 seconds. Cycling parameters for *E. faecalis* were 95°C, 3 minutes; 40 cycles of 64.5°C for 10 seconds. Each species-specific assay was optimized for primer concentration and annealing temperature. *Bacteroides* species assay used 6.25 pm of each primer per reaction with an annealing temperature of 65°C. *E. coli* assays used 6.25 pm of each primer per reaction and an annealing temperature of 63.3°C. *Clostridium* Groups I and XIVa used 7.5 pm of each primer per reaction with annealing temperatures of 58.9°C and 57°C, respectively. All Q-PCR assays included a 6-point standard curve in triplicate ($R^2 > 0.90$) and three no-template controls containing all other reaction components on a Bio-Rad iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, Calif, USA). Three DNA preparations were purchased for the standard curves: *Clostridium perfringens* for *Clostridium* group I (Sigma Aldrich, St. Louis, Mo, USA), *Ruminococcus productus* VPI 4299 for *Clostridium* group XIVa, and *Bacteroides fragilis* VPI 2553 (DNA purchased from American Type Culture Collection, Manassas, Va, USA). Three DNA preparations for the standard curves were prepared in our laboratory by the CTAB method of Ausubel et al. [59] from strains *E. coli* DH5-α, *Enterococcus faecalis* 19433, and *Enterococcus faecium* 19434.

Bio-Rad iQ5 PCR detection system software (Bio-Rad, Hercules, Calif, USA) was used to calculate the Ct value for each reaction, the mean Ct value for each set of triplicates, and the amount of target DNA using values derived from the standard curves. The statistic PV, which quantifies variability in population abundance over time, was calculated for each of the organisms assayed using the Q-PCR data [60].

3. RESULTS

3.1. Dogs enrolled in study

Information on sex, age, breed, and diet of all dogs enrolled in the study is given in Table 1. Five control dogs were identified that lived in MSU colonies, were fed the same food daily, and were exercised indoors. These dogs were from a family developed to study an inherited disorder unrelated to the GI tract. As such, dogs were full or half siblings, or their parents. We also collected feces from four normal dogs living in households to assess variability of the microbiota based on housing and feeding regimes. Of the household pets, two were supervised outside (e.g., walked on leash), one was allowed to roam freely in a suburban environment, and one was allowed to roam freely in a rural

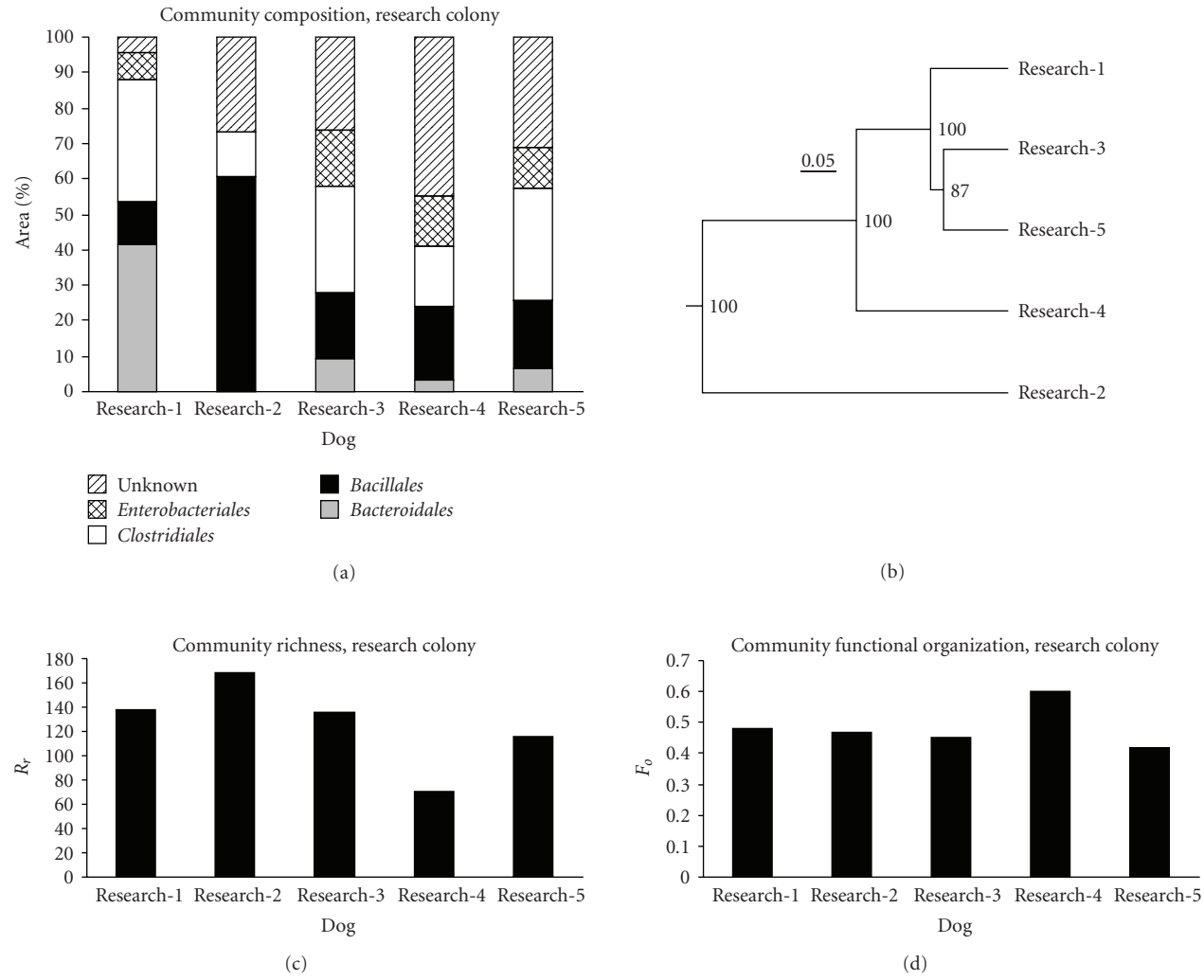

Figure 2: *T-RFLP analysis of fecal samples from research colony dogs*. Panel (a) community composition expressed as % area of peaks under the T-RFLP electropherogram; bacterial groups were combined at the taxonomic level of order. Panel (b) dendrogram based on Bray-Curtis similarities of community composition among the five dogs; numbers at nodes indicate percentage of trees having an equivalent node in jackknife analysis. Panel (c) community richness parameter, *Rr*. Panel (d) community functional organization, *Fo*.

environment. In addition, two of the four dogs living in households experienced diarrhea, one of them twice, after collection of the normal samples. We, therefore, collected samples from these dogs during and after treatment, if any, with the prescribed antibiotic.

Fecal samples were screened for *Giardia* by ELISA. One of two dogs (Pet 8 in Table 1) presenting to the MSU Small Animal Clinic with diarrhea was positive for *Giardia*; all others were negative. Fecal samples were screened for other parasites by fecal flotation; all were negative. DNA was extracted from each fecal sample and characterized using PCR for seven known enteric pathogens. Two of the five research colony dogs carried *Enterococcus faecium, Campylobacter* spp., and *Helicobacter* spp. (Research dogs 1 and 5). Two others of the five carried *Enterococcus* spp. and, *Helicobacter* spp. (Research dogs 2 and 4). The fifth research colony dog carried only *Enterococcus faecium* (Research dog 3). Diagnostic PCR assays performed on fecal DNA obtained from the seven *Giardia*-negative household pets showed that five (Pets 1, 2, 3, 4, and 5) had at least one sample positive for *Enterococcus* spp., *Helicobacter* spp., and *Campylobacter* spp. during the sampling period; all three organisms were detected simultaneously in many samples. Pet 6 was positive for *Campylobacter* spp. and *Enterococcus* spp. but not *Helicobacter* spp. Pet 7, which presented to the MSU Small Animal Clinic with diarrhea, was positive for both *Enterococcus* spp. and *Helicobacter* spp. All thirteen dogs were negative for *Salmonella* spp. and *Brachyspira* spp.

3.2. Bacterial communities in research colony dogs

Results of T-RFLP analysis of fecal samples from research colony dogs are shown in Figure 2. Community composition is shown in Panel (a); for this figure, % areas of peaks corresponding to the bacterial groups assigned by Nagashima et al. [31] were combined at the taxonomic level of order

as follows: *Bifidobacteriales* (peak *Bsl*I 124), *Bacillales* (peaks *Bsl*I 322, 520, and 657), *Bacteroidales* (peaks *Bsl*I 317, 469, and 853), *Clostridiales* (peaks *Bsl*I 370, 494, 749, 919, and 955), *Enterobacteriales* (peak *Bsl*I 940), and Unknown (all peaks not classified in one of the previous groups). While a number of the *Bsl*I peaks defined by Nagashima et al. [31] contain organisms from more than one order, inspection of Table 1 in their publication shows that members of a single genus do dominate almost all peaks. The taxonomic level of order also captures some aspects of the physiology of intestinal bacteria that may be important in determining colonic health.

The fecal bacterial community composition of the genetically related research colony dogs was similar in four of five dogs (Figure 2, panels (a) and (b)). Dogs 1, 3, and 5 are housed in the same room and are the three research animals that were PCR-positive for *Enterococcus faecium*. The values of the community richness measure *Rr* for these communities are shown in Figure 2, Panel (c). These values were calculated using all peaks in each profile, not the combined order level data shown in Panel (a); values were comparable to those reported by Marzorati et al. [57] for microbially rich environments such as soils and sediments. Values of the community functional organization parameter (*Fo* reflects the "evenness" of the pattern of the relative abundances of the organisms) are shown in Figure 2, Panel (d); values ranged from 0.42 to 0.60. Marzorati et al. [57] have characterized *Rr* values >30 as indicating environments capable of sustaining large, diverse communities. *Fo* values for the research colony dog fecal communities fall around 0.45, a value which Marzorati et al. [57] have characterized as indicating a balanced community able to continue functioning properly during perturbations.

3.3. Household pet dogs

Results of T-RFLP analysis of fecal communities from two pet dogs (Pets 1 and 2) living in the same household and followed over time are shown in Figure 3. These dogs were genetically different and were fed different dry diets: an adult diet and a puppy diet from the same manufacturer. The adult dog (Pet 1) was allowed to roam freely in a rural environment, while the puppy (Pet 2) was exercised outdoors under the owners' supervision. The episode of diarrhea in Pet 1 on day 1 was due to dietary indiscretion and resolved by the next day; it was characterized by a "bloom" of *Bsl*I 940 (*E. coli*). Community compositions of fecal samples from the two dogs were dissimilar and exhibited considerable variation over time (Figure 3, Panel (a)). Nevertheless, five of six community composition patterns for Pet 2 did cluster together in the dendrogram shown in Figure 3, Panel (b). Values of the richness parameter *Rr* for Pet 1 ranged from 25 to 50; values of *Rr* for Pet 2 ranged from 11 to 147 (Figure 3, Panel (c)). These *Rr* values were generally lower than those obtained for the research colony dogs. *Fo* values ranged from 0.30 to 0.77 for Pet 1 and from 0.39 to 0.53 for Pet 2 (Figure 3, Panel (d)). *Fo* values for both dogs were more constant than either community composition or *Rr* values. The community dynamics % change parameter was calculated for sequential samples (Figure 3, Panel (e)); because the time intervals between samples were not equal, we did not calculate the rate of change parameter Δ_t. Values for Pet 1 ranged from 0.40 to 0.75 and for Pet 2 from 0.45 to 0.86. These % change values are much higher than those reported for other kinds of microbial communities by Marzorati et al. [57].

One T-RFLP profile was obtained from another household pet (Pet 4); this animal was fed a commercial adult diet and occasional table scraps and allowed to roam freely in a suburban environment; both *Rr* and *Fo* values were low: 6.0 and 0.31, respectively.

3.4. Household pet dog with repeated diarrhea

Results of T-RFLP analysis of fecal samples from a household pet dog (Pet 3) experiencing two separate episodes of diarrhea are shown in Figure 4. This animal was fed a commercial weight control dry diet two to four times higher in fiber than the diets of the other dogs in the study, and was consistently supervised by the owner when outdoors in a suburban environment. At the time of enrollment in the study, this pet was being treated for osteoarthritis with carprofen at 1.0 mg/kg BID; on day 121 after enrollment, the dosage was increased to 2.0 mg/kg BID. Moderate anorexia developed on day 151, and the owner began supplementing the diet in various ways to stimulate appetite; this supplementation continued throughout the rest of the study. After the second episode of diarrhea on day 168, treatment with carprofen was discontinued and treatment with tramadol (2.7 mg/kg TID) was instituted. Tramadol was discontinued due to vomiting on day 219 and deracoxib (1.4 mg/kg SID) was initiated; this drug was also not well tolerated and was discontinued on day 233. No further analgesics were given during the study.

Results of pathogen-specific PCR assays for this animal are shown in Table 3. *Campylobacter* and *Helicobacter* spp. were detected by PCR assay in 11 of 15 samples; the *Campylobacter* spp. PCR assay was positive in two samples taken on the day of onset of the first episode of diarrhea. *Enterococcus faecalis* and *E. faecium* assays were positive following the onset of the first episode of diarrhea. *E. faecalis* was not detected subsequently by this assay, while *E. faecium* levels were below the limit of detection until the onset of the second episode of diarrhea when the dog became positive over a course of 30 days.

Community composition was similar in the normal samples taken on days 1, 17, 70, 78, 85, 113, and 134. Episodes of diarrhea occurred beginning on days 61 and 168; both episodes were treated with metronidazole (1st treatment starting on day 65 (14.0 mg/kg BID, for 10 days) and 2nd treatment starting on day 172 (14.0 mg/kg BID for 6 days)), and a brief period on a low-residue diet. Because the second episode of diarrhea occurred 107 days after the first and the microbiota returned to its starting composition during the interval, the second episode was probably not an antibiotic-associated diarrhea. Community composition returned to its starting composition after the first episode of diarrhea but not after the second; the two episodes were also different in character, the first being dominated by *Enterobacteriales* and *Clostridiales* and the

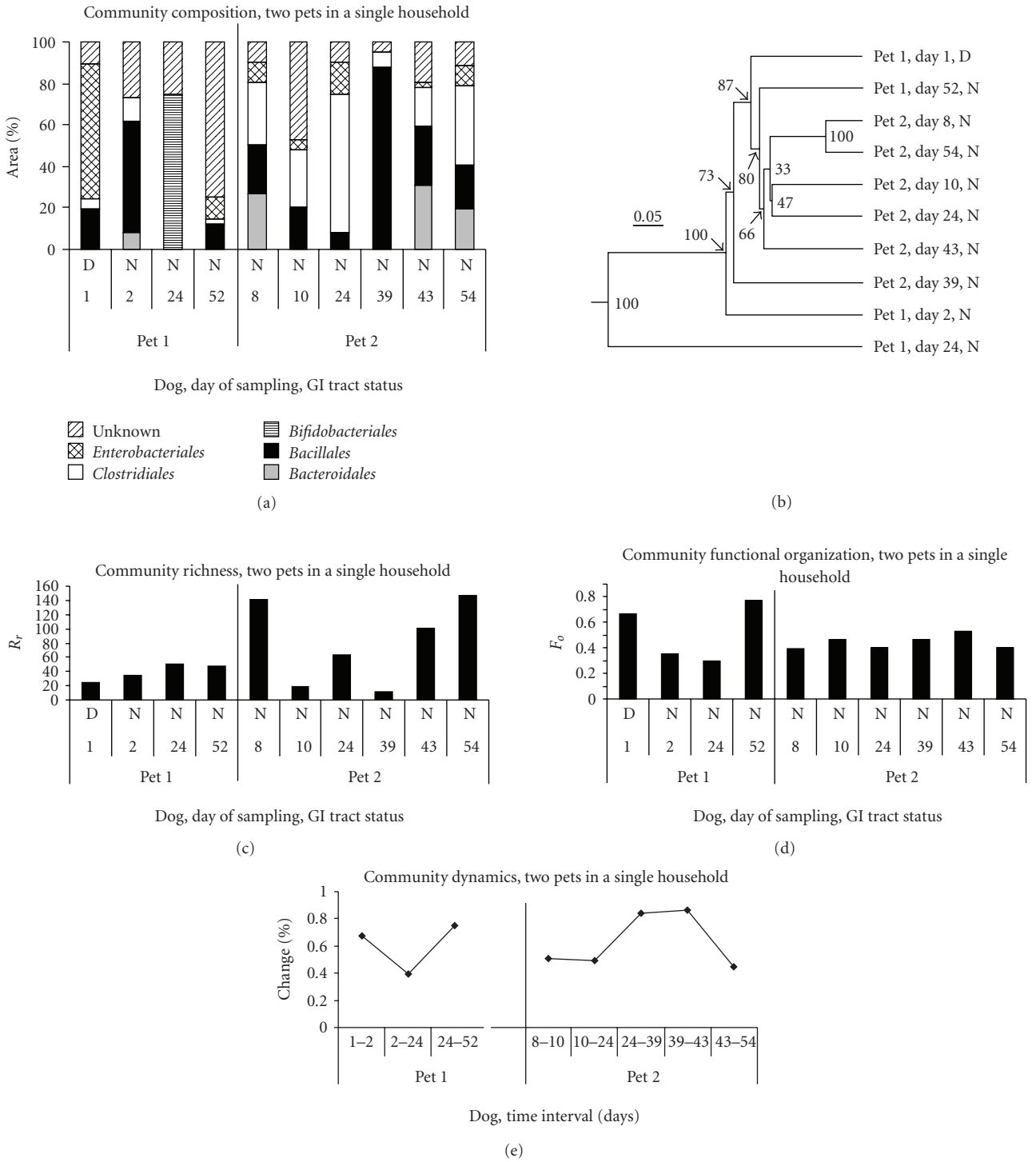

Figure 3: *T-RFLP analysis of fecal samples from two pets in a single household*. Panel (a) community composition expressed as % area of peaks under the T-RFLP electropherogram; bacterial groups were combined at the taxonomic level of order. Panel (b) dendrogram based on Bray-Curtis similarities of community composition among the samples from the two dogs; numbers at nodes indicate percentage of trees having an equivalent node in jackknife analysis. Panel (c) community richness parameter, *Rr*. Panel (d) community functional organization, *Fo*. Panel (e) % change parameter for community dynamics.

second by *Bacillales*. Simple fecal cytology performed by MSU VTH staff at the onset of the second episode of diarrhea was read out as bacterial overgrowth. The community did not begin to return to its previous composition for an extended period after the second episode of diarrhea. On the last day of sampling, representatives of all the orders previously present were detected, but their proportions were altered from those in the normal samples. The dendrogram

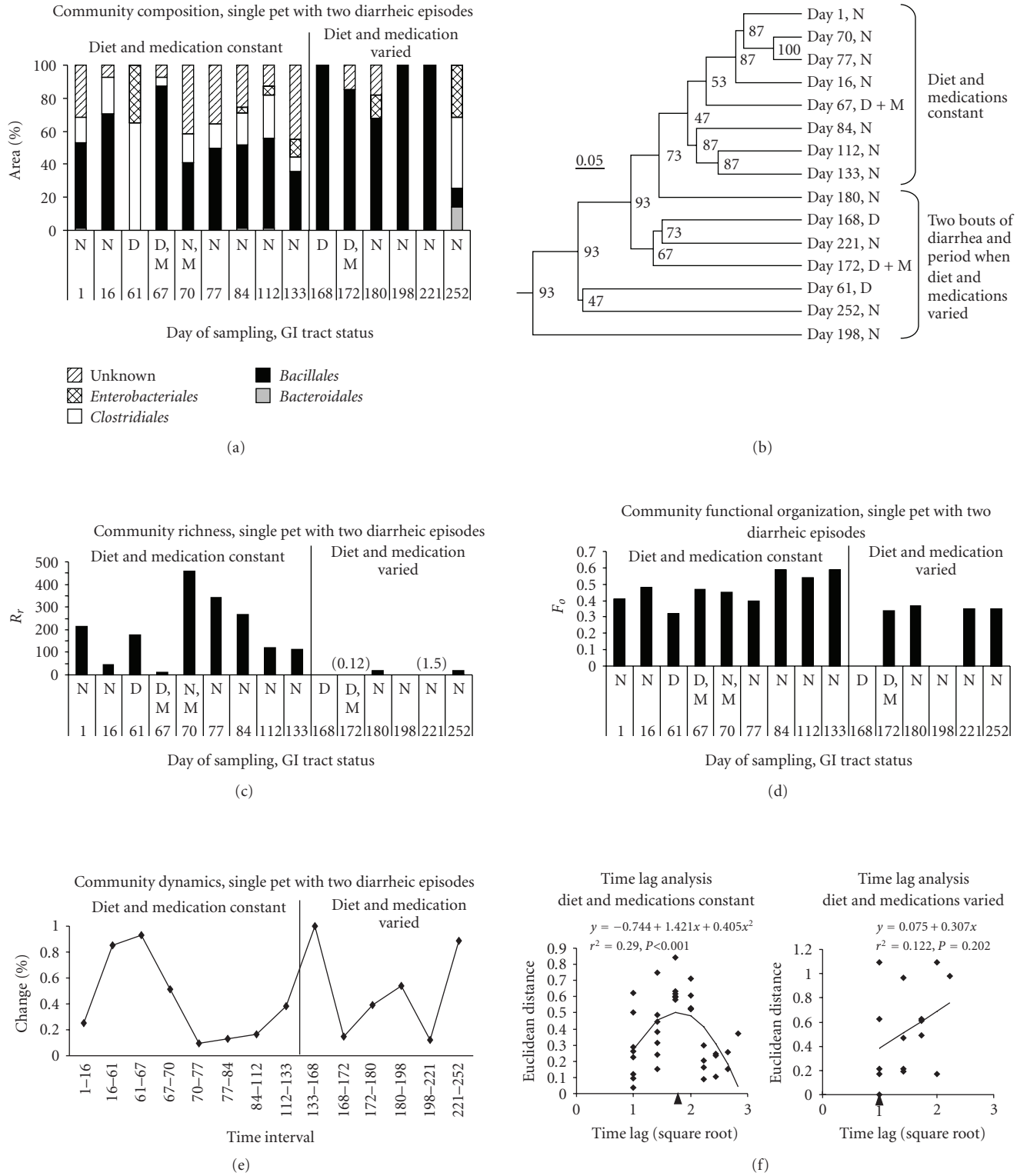

Figure 4: *T-RFLP analysis of fecal samples from a single pet with two diarrheic episodes*. Panel (a) community composition expressed as % area of peaks under the T-RFLP electropherogram; bacterial groups were combined at the taxonomic level of order. Panel (b) dendrogram based on Bray-Curtis similarities of community composition among the samples from this dog; numbers at nodes indicate percentage of trees having an equivalent node in jackknife analysis. Panel (c) community richness parameter, *Rr*. However, *Rr* is not reported when there was only a single peak ≥1% of the area under the electropherogram (days 168 and 198); the value of *Rr* was 0.12 on day 172 and 1.5 on day 221. Panel (d) community functional organization, *Fo*. However, *Fo* is not reported when there was only a single peak ≥1% of the area under the electropherogram (days 168 and 198). Panel (e) % change parameter for community dynamics. Panel (f) time lag analysis. Arrows below the *X*-axis indicate the day of onset of an episode of diarrhea.

Table 3: Detection of potential pathogens in pet dog with two diarrheic episodes by standard PCR assay*.

Day	Status**	*Enterococcus faecalis*	*Enterococcus faecium*	*Campylobacter* spp.	*Helicobacter* spp.	*Clostridium perfringens*
1	N	−	−	+	+	−
16	N	−	−	−	+	−
61	D	−	−	+	+	+
67	D, M	+	+	−	−	+
70	N, M	+	+	−	−	−
77	N	−	−	−	−	+
84	N	−	−	−	+	+
112	N	−	±	−	+	−
133	N	−	−	−	+	−
168	D	−	+	−	−	+
172	D, M	−	+	−	+	+
180	N	−	+	−	+	+
198	N	−	±	−	+	+
221	N	−	−	−	+	+
252	N	−	−	−	+	+

*"−": negative; "+": positive; "±": weak positive. All fecal flotation and *Giardia* tests were negative.
**N: normal; D: diarrhea: M: metronidazole treatment.

showing the similarities of the communities at different times (Figure 4, Panel (b)) also demonstrates this pattern: most of the samples taken prior to day 168 cluster together and away from the diarrheic samples and the normal samples taken after the second episode of diarrhea.

Changes in community richness parameter *Rr* values are shown in Figure 4, Panel (c). Community richness dropped to a value of 11.2 following institution of metronidazole treatment for the first episode of diarrhea but rebounded to a high level (460) and then declined slowly over the next month. Richness was severely reduced during the second episode of diarrhea and did not return to even moderate levels; repeated changes in medications and diet were made during the period after the second episode of diarrhea. Changes in *Fo* values (Figure 4, Panel (d)) were less pronounced but consistently somewhat lower after the second episode of diarrhea.

Changes in the community dynamics parameter % change also reflect the events described above (Figure 4, Panel (e)). Large values (0.85 to 0.99) are associated with the two episodes of diarrhea. Prior to the first episode of diarrhea and after recovery from it, the % change values ranged from 0.09 to 0.38 and were comparable to those reported for other kinds of communities by Marzorati et al. [57]. Marzorati et al. characterized % change values around 0.10 as indicating that the community is stable; new organisms are able to become established but do not interfere with community function. Very high % change values were associated with the diarrheic episodes in Pet 3, and after the second episode, the % change values continued to fluctuate, indicating that the community did not return to stability. This period of instability coincided with the period of repeated changes in medications and diet.

Temporal variability in the T-RFLP data was also analyzed using the regression method of Collins et al. [58] (Figure 4, panel (f)); the period when diet and medication were constant and the period when both varied were analyzed separately. The period when diet and medications were constant, which included the first episode of diarrhea, exhibited a phase with a positive slope followed by a phase with a negative slope. This pattern can be interpreted as showing a directional change in community composition after a perturbation followed by a return to the starting condition [58]. The period when diet and medications were varied did not yield a significant regression; according to Collins et al. [58], this result indicates fluctuating changes in community composition over time.

3.5. Q-PCR analysis of fecal samples from a household pet dog experiencing repeated episodes of diarrhea

To explore further the results for Pet 3 obtained from T-RFLP analysis, we performed quantitative real-time PCR on the same DNA samples used for T-RFLP. Q-PCR results are shown in Figure 5, Panels (a) to (f). Population variability (PV) values were high (range: 0.768–0.915) for all organisms assayed. *Clostridium* groups I and XIVa were analyzed separately; group I contains the potential enteric pathogen *C. perfringens*. (*C. difficile* is a member of *Clostridium* group XI and would not be detected by these assays.) Both groups were detected by Q-PCR throughout the sampling period. *Clostridium* group I levels were relatively constant, except for an 880-fold increase coincident with the first episode of diarrhea (day 61); the *Clostridiales* peaks comprised 55% of the total area under the electropherogram for that sample in the T-RFLP analysis (Figure 4(a)), and the *Bsl*I 749 peak comprised 40% of the total area under the electropherogram (data not shown). The *Bsl*I 749 peak is included in the *Clostridiales* portion of the bar in Figure 4(a). The expected

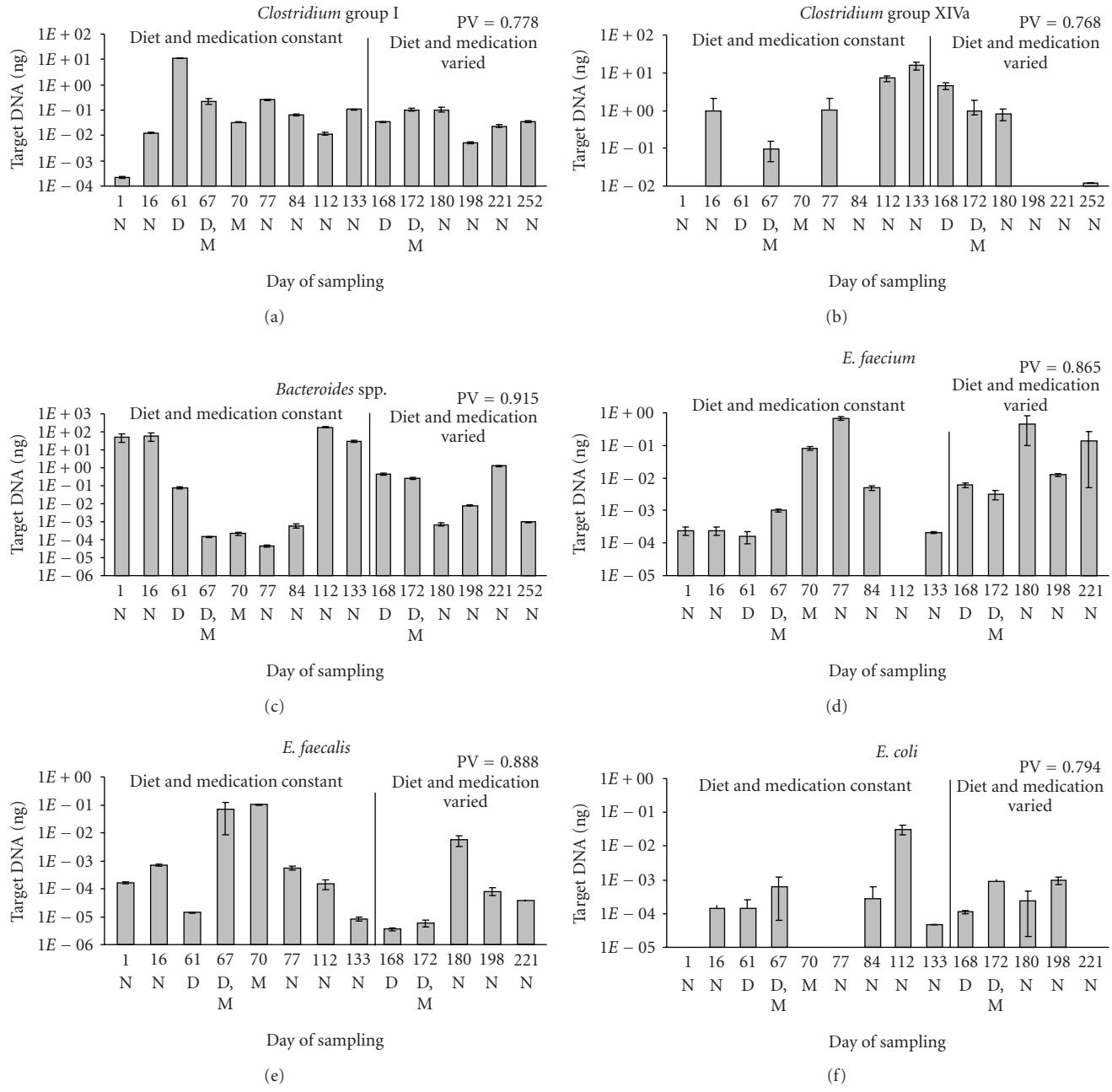

Figure 5: *Q-PCR analysis of bacterial groups in fecal samples from a single pet with two diarrheic episodes*. Panel (a) *Clostridium* group I; Panel (b) *Clostridium* group XIVa; Panel (c) *Bacteroides* spp.; Panel (d) *E. coli*; Panel (e) *E. faecium*; Panel (f) *E. faecalis*. R^2 values for the standard curves were 0.995, 0.986, 0.956, 0.958, 0.980, and 0.998, respectively. Error bars indicate the standard deviation of triplicate samples. Lack of a bar indicates that no product was detected in 40 cycles. The sample for day 252 was exhausted before Q-PCR for *E. faecium, E. faecalis,* and *E. coli* was performed. Population variability (PV) values are shown at the upper right of each panel.

*Bsl*I peak size for *Clostridium perfringens* is approximately 750 bp. Once this result was obtained, we performed a specific PCR assay for *Clostridium perfringens* [45]. That assay was negative prior to the first episode of diarrhea, it became strongly positive in the first diarrheic sample (Table 3), and remained so in all but one sample for about three weeks. Four weeks after the last positive sample, the assay was negative and remained so until the onset of the second episode of diarrhea, when it became strongly positive again. It remained positive throughout the second metronidazole treatment and was still positive twelve weeks later.

Clostridium group XIVa levels were more variable and did not exhibit any pattern with respect to either episode of diarrhea. Levels of *E. coli* exhibited a peak on day 112 but also did not exhibit any pattern with respect to either episode of diarrhea.

Levels of *E. faecium* and *E. faecalis* both rose after the initiation of metronidazole treatment during both episodes

of diarrhea. *E. faecium* rose 4000-fold between the onset of the first episode of diarrhea (day 61) and day 77 and 77-fold between the onset of the second episode of diarrhea (day 168) and day 180. *E. faecalis* rose 7000-fold between the onset of the first episode of diarrhea (day 61) and day 70 and 1500-fold between the onset of the second episode of diarrhea (day 168) and day 180.

Q-PCR assay detected *Bacteroides* in all samples. Levels of *Bacteroides* spp. were highest in normal samples taken during the period when diet and medications were constant. *Bacteroides* spp. decreased approximately 10^5-fold during and following the first episode of diarrhea but returned to the original levels. Levels decreased approximately 10^4-fold during the second episode of diarrhea and fluctuated thereafter. This result is in marked contrast to the T-RFLP assay, which seldom detected *Bacteroides* at levels exceeding 1% of the total area under the electropherogram.

4. DISCUSSION

Screening for specific pathogens by PCR revealed that most animals carried one or more potential enteric pathogens in their fecal microbiota even when they had no clinical signs. Thus, many cases of "spontaneous" diarrhea in dogs—and by extension, sporadic diarrhea in humans—may be caused by organisms already present in the GI tract following perturbation of the microbiota by an environmental factor rather than by a pathogen acquired from another source [6, 61]. The hypothesis that alterations in the microbiota may inhibit or facilitate disease processes has been invoked in the context of chronic inflammatory bowel diseases; our results support the idea that there may be a significant role for the microbiota in acute infectious disease processes. Other clinical and experimental studies suggest that the relative balance of aggressive and protective bacterial species is altered in inflammatory diseases such as Crohn's disease (CD), ulcerative colitis (UC), and pouchitis. In a review of current work in this area, it was postulated that overly aggressive immune responses to a subset of commensal (nonpathogenic) enteric bacteria in genetically predisposed individuals result in disease [62]. Recently, Frank et al. showed that CD patients, UC patients, and noninflamed controls had statistically significantly different microbiotas based on culture-independent rRNA sequence analysis of cloned libraries [63]. Because it was based on surgical samples of colon, this study provided a survey of gut-wall associated microbiota relevant to Inflammatory Bowel Disease. Here, as in all studies using methods based on SSU rRNA, bacterial "numbers" were recognized to be relative estimates reflecting gene copy numbers and not indicative of causation. However, microbiota surveys can provide candidates for hypothesis testing of causation. In our study, fecal samples could be argued to best represent "diagnostic" samples that would be taken from a host presenting with acute diarrhea. It is acknowledged that further work will be needed to document the ability of readily available fecal samples to represent different locations within the colon to document the etiologies of these diarrheas [64].

The influence of diet and medication on the intestinal microbiota has been studied directly in animals and humans [17, 18, 21, 29, 62]. Although it was not our intent to study such effects, our results confirm that the composition of the intestinal microbiota is quite sensitive to changes in environmental factors such as changes in diet and/or medications as well as exposure to the microbiotas of other animals. The relatively consistent microbiotas of the research colony dogs are in marked contrast to the fluctuations observed in Pet 1, which roamed freely in a rural environment. The microbiotas of Pets 2 and 3, which were more closely supervised when out of doors, showed less variability than that observed in Pet 1 but more than in the research colony dogs. Finally, fluctuations in the microbiota of Pet 3 became much more pronounced when diet and medication were changed.

Comparison of results from T-RFLP and Q-PCR results for Pet 3 showed that both methods can detect variation in the microbiota associated with events such as diarrheal episodes and changes in diet and medication. The extensive variability in the abundances of the different groups of organisms evident in the T-RFLP data was also evident in the Q-PCR results for individual groups and organisms; population variability (PV) values for all organisms assayed by Q-PCR were high. This result is not surprising given the wide range of target DNA concentrations detected by this assay (lowest detected level, 3.6×10^{-6} ng for *E. faecalis*; highest detected level, 1.8×10^2 ng for *Bacteroides*).

The T-RFLP analysis indicated that there was a large increase in *Clostridiales,* including the *Bsl*I 749 bp peak defined by Nagashima et al. [31], on day 61, the day of onset of the first episode of diarrhea. Q-PCR analysis indicated an increase in *Clostridium* group I, of which *C. perfringens* is a member, which coincided with the onset of the first episode of diarrhea. Diagnostic PCR assays for *C. perfringens* showed that this organism was temporally associated with the onset of both episodes of diarrhea. But because the time intervals that elapsed between samples were long, we cannot determine whether the increases in *Clostridium* group I or the detection of *C. perfringens* reflected causes or consequences of the episodes of diarrhea. The data thus suggest but do not prove that the illness was caused by a member of *Clostridium* group I, possibly *C. perfringens*. However, *C. perfringens* remained at detectable levels during the period when diet and medications varied. This result suggests that the instability of the microbial community during this period facilitated the growth of this potential pathogen.

T-RFLP analysis also indicated large increases in *Bacillales;* which includes lactobacilli, streptococci, and enterococci; subsequent to metronidazole treatment. In Q-PCR analysis, both *E. faecium* and *E. faecalis* exhibited repeated substantial increases after the initiation of metronidazole treatment, and after the second episode of diarrhea, *E. faecium* levels did not return to those seen at the beginning of the study. These results suggest that, in view of the potential of both *E. faecium* and *E. faecalis* to have deleterious effects on the GI tract, the use of metronidazole as a first-line treatment for canine diarrhea should be re-evaluated.

Perturbations in *Bacteroides* spp. levels associated with the two episodes of diarrhea were also apparent; levels of these organisms decreased during both episodes of diarrhea and became unstable during the period when diet and medication were varied. Since *Bacteroides* spp. are major components of the colon microbial community and essential to its function, such fluctuations might be expected to have repercussions for colon health.

The discrepancy between the two methods in detecting the genus *Bacteroides* in this animal would be most simply explained as a relative inability of the universal eubacterial 16S primers used to bind to the 16S rDNA sequences of the particular *Bacteroides* spp. predominating in that dog compared to the more specific primers used in the Q-PCR assay. However, members of the *Bacteroidales* were detected by the primers used in T-RFLP analysis in four of the five research colony dogs and in Pets 1 and 2. BLAST searches indicated that the region of homology between the Nagashima et al. [31] reverse primer to the 16S rDNA sequence of (1) some but not all strains of *Bacteroides eggerthii*, (2) some but not all strains of *B. stercoris*, and (3) some but not all strains of *B. caccae* consists only of the seven bases from nucleotide 11 to nucleotide 17 of the 19-base primer. This limited homology was even present in the 16S rDNA sequences of the type strains of *B. stercoris* and *B. caccae*. No PCR product would be obtained from DNA of these strains. Also, BLAST searches indicated that the 16S primers used for the Q-PCR studies [51] would be expected to amplify rDNA of all strains of these three species. However, *B. eggerthii, B. stercoris*, and *B. caccae* are found in humans; in a cloned library study, sequences closely related to *B. stercoris* were obtained from dogs [20]. Of the *Bacteroides* spp. sequences obtained from dogs in the latter study, one was very similar to that of *B. stercoris*, three were similar to that of *B. vulgatus,* and four were not similar to those of any of the published *Bacteroides* spp. used in the analysis. The same study also indicated that the *Bacteroides* spp. sequences from the fecal microbial communities of humans, dogs, cats, and gulls clustered together and separately from those of cattle and elk.

We applied the community characterization schema proposed by Marzorati et al. [57] to the dog fecal bacterial communities studied here and related them to what we know about the management and clinical presentation of the dogs. The research colony dog communities were characterized by high richness and intermediate "balanced" levels of functional organization. Because of the constancy of the environment of these dogs, we predict that their communities would experience low-to-medium dynamics similar to those observed in normal samples from Pet 3. The communities of Pets 1, 2, and 4 were generally characterized by lower richness, balanced functional organization, and high levels of dynamic change for Pets 1 and 2.

The normal communities of Pet 3 prior to and following the first episode of diarrhea were characterized by high richness, medium levels of dynamic change, and balanced functional organization, while the diarrheic samples were characterized by low richness, high levels of dynamic change, and low levels of functional organization. Communities in the normal samples taken following the second episode of diarrhea were characterized by continued low richness, fluctuating levels of dynamic change, and somewhat lower levels of functional organization than previous normal samples. The fluctuating levels of dynamic change were temporally correlated with changes in diet and medication. Time lag analysis indicated that samples taken when diet and medication were constant—the initial samples, samples taken during the first episode of diarrhea, and samples taken prior to the second episode of diarrhea—showed a recognizable pattern of disturbance followed by a return to the initial condition. Samples taken when diet and medications were varied—beginning with the onset of the second episode diarrhea and continuing to the end of the study—did not exhibit any directional changes but instead showed random fluctuation. The general agreement between these complementary analyses suggests that concepts from macroecology will be useful in interpreting data from microbial communities.

According to Marzorati et al. [57], the level of the community richness parameter *Rr* is indicative of the carrying capacity of the environment; diet is one obvious environmental variable that might affect the carrying capacity in the GI tract. The similar values of *Rr* obtained for research colony dogs fed and housed under controlled conditions supports this idea. However, Pet 2 was genetically related to the research colony dogs and was fed a similar diet, but exhibited lower community richness. This free-roaming dog probably had a much more variable intake than the research colony dogs, so values for apparent carrying capacity might have been influenced by the highly dynamic nature of the microbial community in this animal. A similar effect can be seen in the richness parameter values for Pet 3; *Rr* varied widely subsequent to perturbation and periods of dynamic change in the community due to episodes of diarrhea.

The level of dynamic change also varied considerably in Pets 1, 2, and 4, and was correlated with environmental factors in that Pets 1 and 2 experienced both more environmental variation and consistently higher levels of change in community composition than did Pet 3 during healthy periods. The community functional organization parameter *Fo* is held by Marzorati et al. [57] to reflect the resistance of the community structure to perturbation. If *Fo* values do predict community resiliency, then such resiliency may explain why both Pet 1 and Pet 2, which had relatively robust *Fo* values, were mainly non-diarrheic in spite of having high levels of dynamic change and substantially shifting community compositions. In the case of Pet 1, the latter phenomena may have been due to varied intake of substances from the environment; in the case of Pet 2, a puppy, these phenomena may have been due to maturation processes in the GI tract. In addition, after the second episode of diarrhea in Pet 3, *Fo* values declined and the community appeared to become less stable under the influence of changing diet and medication regimes; this observation further supports the connection between functional organization and resiliency.

Studies with larger sample sizes are required to substantiate these apparent correlations in a rigorous way. Also, more work is clearly needed to delineate the variability of

the microbiota of the healthy GI tract before embarking on detailed studies of disease states. However, based on these observations, we would predict that if the normal or background variability is substantial, it may prove exceedingly difficult to detect relevant changes in heterogeneous populations such as individuals enrolled in clinical trials.

5. CONCLUSIONS

We hypothesized that dogs have a stable composition of the colon microbial community and that episodes of diarrhea lead to long lasting changes in community composition and/or function; furthermore, treatment for specific pathogens can compound these effects. Outbred monogastric animals like dogs can serve as easily manipulable models to address approaches for problems of the human GI tract. Thus, the microbiota of dogs was studied during diarrheic episodes and compared to those of healthy control dogs to make a preliminary assessment of the contribution of members of the microbiota to acute diarrheal disease processes. We found that (1) four of five dogs living in an environment expected to provide the least exposure to factors that might alter the GI tract microbiota had similar microbiotas, (2) the microbiotas of dogs kept in more variable environments were correspondingly more variable, (3) acute episodes of diarrhea resulted in large-scale changes in the GI tract microbiota, and (4) when the diet and medications of a dog having a previously stable microbiota were changed repeatedly, the GI tract microbiota also changed repeatedly, ultimately reducing richness. The high levels of variability we encountered in the pet dogs indicate that descriptive population-based microbiota studies may be so fraught with variation within and between individuals that meaningful patterns and changes may be hard to distinguish from the "noise." Either longitudinal studies of individuals under relatively constant environmental regimes (Pet 3) or model-based studies of groups of individuals under strictly controlled environments (research colony dogs) with planned experimental interventions could be expected to yield interpretable results. The consistency of the microbial communities in the research colony dogs and the changes we were able to observe in Pet 3 indicate that it is possible to establish baseline starting conditions and that the methods employed in these studies can be used to detect and delineate changes in fecal microbial communities. We expect these considerations derived from this useful animal model to apply with equal force to studies in humans.

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Review Article

Emerging Insights into Antibiotic-Associated Diarrhea and *Clostridium difficile* Infection through the Lens of Microbial Ecology

Seth T. Walk[1] and Vincent B. Young[1,2]

[1] *Division of Infectious Diseases, Department of Internal Medicine, University of Michigan Health System, Ann Arbor, MI 48109, USA*
[2] *Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48109, USA*

Correspondence should be addressed to Seth T. Walk, sethwalk@umich.edu

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Antibiotics are the main, and often only, clinical intervention for prophylactic and active treatment of bacterial infections in humans. Perhaps it is not surprising that these drugs also shift the composition of commensal bacteria inside our bodies, especially those within the gut microbial community (microbiota). How these dynamics ultimately affect the function of the gut microbiota, however, is not fully appreciated. Likewise, how antibiotic induced changes facilitate the outgrowth and pathogenicity of certain bacterial strains remains largely enigmatic. Here, we discuss the merits of a microbial ecology approach toward understanding a common side effect of antibiotic use, antibiotic-associated diarrhea (AAD), and the opportunistic bacterial infections that sometimes underlie it. As an example, we discuss how this approach is being used to address complex disease dynamics during *Clostridium difficile* infection.

1. INTRODUCTION

The human colon contains the most abundant and diverse assemblage of bacteria in the body. Symbiotic interactions with and within this complex community are now recognized as important predictors of human health. Aberrant community structures are associated with complex diseases like obesity, irritable bowel syndrome, and immune dysfunction. Antibiotic administration can disrupt the colonic ecosystem, which, in turn, leaves patients vulnerable to gastrointestinal disease. Diarrhea is a common manifestation of antibiotic-mediated disturbance and can result from altered function of the disrupted microbiota, direct effects on host tissue, and colonization by opportunistic organisms that invade the altered microbial community. Here, we review the relevant microbial ecology of antibiotic-associated diarrhea with an emphasis on bacterial community dynamics during *C. difficile* infection.

2. COMMONALITIES AND ASSUMPTIONS FOR GI TRACT MICROBIAL ECOLOGY

When initiating a discussion of the microbial ecology of the gastrointestinal (GI) tract, it is important to review some of the common areas and assumptions investigators used when studying this ecosystem. First, the proportion of uncultivable bacteria in the GI tract is high (~60%–80%). Initially, culture-based surveys of the gut microbial successfully isolated and characterized large numbers of the bacterial morphotypes (i.e., distinct cellular forms) present in human feces [1, 2]. However, recent surveys based on DNA sequencing have indicated that the vast majority of genetically distinct organisms have not been isolated by culture techniques [3]. These relatively new sequence-based approaches in combination with robust bioinformatics provide the framework to explore a vast amount of genetic diversity. It is now feasible to survey nearly all of the genetic information in a given system,and this ability has ushered in

a new area of research, referred to as metagenomics [4]. The field is still in its infancy, and much of the data continue to be open for interpretation. It is important to note that the currency for GI tract microbial ecology in the metagenomic era is the abundance and distribution of targeted DNA sequences and not actual organisms or randomly sampled genomes of organisms. The amplification, cloning, and sequencing of certain loci, such as the highly conserved 16S rRNA locus, are the tools used to study the phylogenetic signal contained in the metagenome, and this is different than classical metagenomics, where one seeks to analyze the functional and sequence-based diversity contained in all microbial genomes of communities [4, 5]. Lastly, we draw attention to an early few studies that use culture-based approaches, but will put these data into a metagenomic context.

There are measurable, statistical, and real differences (i.e., not all the detectable differences are biologically significant) between the bacterial communities throughout the human body (skin, mouth, vagina, GI tract, etc.). Studies have shown regional differences in microbial composition throughout the mammalian GI tract in both the longitudinal (i.e., stomach to small intestine to large intestine) and axial (i.e., mucosal associated to mucus to lumen) directions [6–8]. For further discussion on this topic, see the recent review by Peterson et al. [7]. Currently, most studies circumvent the practical and ethical problems associated with direct intestinal sampling (e.g., via colonoscopy and biopsy) by using feces as a proxy [9]. Many of the studies reviewed here do the same and regard the bacterial community in feces as representative of the gut microbiota as a whole, with the caveat that existing spatial community differences may result in a biased representation. For example, total anaerobe counts were found to be 100 times lower in the human cecum compared to feces [10].

Lastly, it is generally assumed that the abundance and distribution of an organism (16S rRNA gene sequence) and broader taxonomic groups of organisms (sequences grouped based on percent similarity and called operational taxonomic units or OTUs) are important. The abundance and distribution of OTUs are often called community structure. As we will discuss in detail below, there are observable patterns in the gut microbiota under certain conditions. Some taxonomic groups are very abundant, while others are at such low abundance that they can only be detected using highly sensitive and specific molecular techniques. Most studies look for community structure and try to assess the underlying mechanisms that caused it (disease, diet, drug effect, etc.). While this may at first seem logical and perhaps trivial, it is currently not well understood what these patterns really mean. For example, what OTUs should be used to assess structure? At the phylum level, patterns may be clear, but at the species level, where functional variation is driven by evolutionary processes, the structure may not be statistically different from a random assemblage (due, in part, to the lack of a universal bacterial species concept [11]). Currently, a challenge for microbial ecologists is to understand dynamics with respect to the functional attributes of bacterial communities and not only through the lens of taxonomy.

3. NORMAL GUT MICROBIOTA

The human colon is typically associated with 10^{11} to 10^{12} bacterial cells per gram of contents, and new estimates using genetic diversity suggest that the gut ecosystem holds 15000–36000 different species [9, 12, 13]. Colonization normally begins at birth, and a variety of bacteria can be detected in infant stools within the first few days after vaginal delivery [14]. Among the first gut bacteria to colonize infants were *Escherichia coli* and *Staphylococcus aureus* [15, 16]. These studies used culture-based methods to show that abundance was highest at about one week after birth and decreased 1–3 orders of magnitude within the first year, suggesting that the abundance of early bacterial colonizers is subsequently shifted by the growing biologic complexity of this system. Recently, nonculture-based data supported these findings and showed that multiple shifts occur among different taxonomic groups over the first 200 days of life [17]. Also, the gamma-Proteobacteria, to which *E. coli* belongs, appear to be the dominant members in these infant's GI tract. It is interesting to note that *E. coli* was initially discovered in 1884 and studied by the famous German pediatrician Theodor Escherich because of its presence in "normal" infant microbiota and because of its beneficial effects on digestion [18].

Defining normal gut microbiota is challenging because of the compositional heterogeneity that exists between hosts [19]. Most phylotypes (suspected species) are unique to the individual being sampled [3]. At broader taxonomic levels, a consistent community structure is often observed, leading to the conclusion that the gut is dominated by members of a few bacterial phyla (Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria). The human gut is described as "exclusive" because there are more divisions (phyla) of bacteria and archaea known to exist on earth than what is typically sampled from human subjects (currently the Silva 16S rRNA database has 115 bacterial divisions of which only 10 have been sampled from humans) [7].

The bacteria in our GI tract are important for certain aspects of human health, and there are clear mutualisms between human and bacterial cells [20]. Not surprisingly, our immune system defends against negative symbiotic interactions basedon prior exposure and also on stimulating mechanisms like breast feeding and vaccinations (prior exposure to living cells is not always necessary for an effective immune response). Some of the traits that make us human also dictate the structure of the gut community, as the microbiota of conspecific relatives (same species of humans, primates, and nonprimates) was most similar to each other in a recent study [21]. There are few data that describe the community structure of the GI tract microbiota in healthy individuals and this limits our ability to formulate generalities on the normal state. However, if we are to consider the healthy human gut as a theoretically-based community, where a consistent structure is defined and used to test hypotheses, then the microbiota of individuals

should converge upon a similar structure under similar conditions [22]. In the absence of convergence, we are left to the study of stochastic events and patterns that are best explained by random walk models, where species traits do not correlate with the abundances along environmental gradients (for more on the theoretical issues concerning community analysis, see Tilman [22]).

Because of the low degree of similarity between individuals, changes in the gut microbiota are typically measured by shifts in structure. For example, a cohort study of 1032 infants showed that breast-fed infants have a consistently different bacterial composition than bottle-fed infants [23]. Based on real-time PCR and OTU specific probes, formula-fed infants ($n = 232$) were colonized by *E. coli*, *C. difficile*, *B. fragilis*, and lactobacilli more often than breast-fed infants ($n = 700$). Similar comparative studies have shown associations between an altered gut microbiota and a number of human diseases, including obesity [24], Crohn's disease [25], irritable bowel syndrome [26], and allergies [27]. It is clear that our understanding of the normal gut microbiota is limited and just beginning, but comparative studies like these illustrate a novel ability to describe the microbial ecology that underlies many complex diseases.

4. ANTIBIOTICS INCREASE HOST SUSCEPTIBILITY TO PATHOGENS

One measure of ecosystem stability, in terms of maintaining function [28], is the ability to resist invasion and subsequent dominance by immigrating organisms. For the gut ecosystem, antibiotic therapy represents a strong perturbation that shifts the relative proportion of community members, allowing opportunists to establish [29–32]. Antibiotic therapies exclude members of the community by eradicating them directly or indirectly by breaking necessary mutualistic interactions [33]. During such events in murine models, the community structure was disrupted and enteric pathogens reached high numbers [34, 35]. Similar observations underlie the proposed colonization resistance or barrier function, provided to the host by the gut microbiota [32, 36, 37], preventing the ingress of pathogens into the gut ecosystem.

Many details about the colonization resistance function of the microbiota have yet to be tested, but it is clear that shifts in the gut microbial community structure are permissive to the establishment of certain pathogens. For example, *Vibrio cholerae* does not normally cause disease in conventional guinea pigs, but it established and caused severe disease after disruption of the microbiota by pretreatment with streptomycin [38]. Similarly, it has been shown that mice with a conventional gut microbiota require a much higher infective dose (10^9 colony forming units per mL, CFU/mL) for colonization by a gram-negative bacterium compared to antibiotic treated mice (10^2 CFU/mL) [39]. The mechanisms behind colonization resistance in humans are topics of ongoing research, but the gut microbiota in animal models has been shown to (i) utilize essential nutrients before they are available to invading bacteria (resource limitation), (ii) limit access to attachment sites (space limitation), and (iii) produce inhibitory substances [40].

Many factors, including drug dose, route of administration, absorption, and host inactivation, dictate the intensity of antibiotic effects on the gut microbiota (see review by Sullivan et al. [32] for specific effects of commonly used drugs). A number of culture-based and nonculture-based molecular techniques have been used to follow bacterial community dynamics in humans upon exposure to antibiotics. Often, specific groups of OTUs are singled out with specific probes. Temporal effects of antibiotic treatment were recently shown among members of the Bacteroidetes division using culture techniques and genetic fingerprinting (rep-PCR) [30]. During a case-control study of subjects taking capsules of 150 mg clindamycin (orally), each individual was sampled prior to antibiotic treatment and at set time points throughout the following 2-year posttreatment. The overall diversity of this division decreased upon antibiotic treatment and remained reduced during the entire 2 years of the study. The authors also show that the dominant community members changed markedly in relative abundance during the first 3 weeks of the posttreatment, suggesting that these effects were not exclusive to the rest of the microbiota.

We draw attention to these dynamics here to simply point out that the gut microbiota changes markedly during and after normal therapeutic courses of antibiotics and that host susceptibility to subsequent infection is increased as a result. We now turn to specific clinical presentations that result from antibiotic treatment of human patients and follow with a discussion on a microbial ecology approach to these diseases.

5. ANTIBIOTIC-ASSOCIATED DIARRHEA AND *C. DIFFICILE*

Patients undergoing antibiotic treatment often develop diarrhea (antibiotic-associated diarrhea or AAD) as a side effect of therapy. Approximately, 5%–25% of patients on antibiotic therapy develop AAD, which can range from a mild, self-limiting illness to a serious and progressive pseudomembranous colitis [41, 42]. The risk of developing disease is highly variable and depends on host factors (age, diet, immune system function, etc.), the type and dose of antibiotic, and the duration of treatment. In a cohort study, Beaugerie et al. found that 17.6% (46 out of 262) of adult ($\geq$18 years old) outpatients developed diarrhea within 14 days after the start of treatment [43]. Patients that remain in the hospital are similarly affected. According to a prospective study of hospitalized patients in Sweden, 12% (294 out of 2 462) of patients $\geq$12 years old developed diarrhea within 45 days after the start of treatment [44]. However, certain patient populations in the hospital appear to be at an elevated risk as 60% (9 out of 15) of individuals (ages 37–79) enrolled in a cohort study of intensive care units developed diarrhea within the first week after of antibiotic treatment [45]. These data illustrate that diarrhea is a common complication of antibiotic use and suggest that critically ill patients are exquisitely susceptible to AAD.

An etiologic agent is not necessary for AAD, as certain drugs can cause gastrointestinal dysfunction directly [42]. A distinction can, then, be made between pathogen-associated and pathogen-independent AAD in that Koch's postulates are not met in the classical sense. For example, if the bacteria responsible for breaking down fermentable starches in the colon are eliminated by the effect of an antibiotic, an osmotic diarrhea may present. In this scenario, the community and not a defined pathogen is responsible for the disease etiology. To our knowledge, however, replicating the disease in an otherwise naïve individual by establishing the "pathogenic community" has not been shown.

A number of opportunistic pathogens can cause disease during antibiotic therapy, including *Salmonella* spp., *Clostridium perfringens*, *Klebsiella oxytoca*, *S. aureus*, *Candida albicans*, and *C. difficile*. Of these, *C. difficile* is the most common cause of pathogen-associated AAD (15%–25%), the most common cause of severe disease, and it causes nearly all cases of nosocomial pseudomembranous colitis [46]. *C. difficile* is an anaerobic, spore forming bacterium that is commonly found in soil, humans, and animals [47]. This toxigenic gram-positive bacillus is asymptomatically carried by 1%–3% of the human population, but is more prevalent among infants [23], hospitalized patients (55.4% of the hospital population in the Swedish AAD cohort study mentioned above [44] were positive for *C. difficile* toxin), older (≥60 years) patients [47–49], and healthcare personnel that care for patients being treated with antibiotics [50]. This pathogen can cause disease in nonhospitalized patients [51], where the main risk factors are antibiotic therapy, proton pump inhibitors, and the use of histamine-2-receptor antagonists [52].

Pseudomembranous colitis in the distal colon and rectum is fatal in 6%–30% of cases [47]. Disease onset occurs several days to several weeks after initial antibiotic treatment and certain drugs, such as clindamycin, cephalosporins, fluoroquinolones, and β-lactams, are associated with greater risk of CDAD [46, 53]. Oral antibiotic therapies with vancomycin, metronidazole, bacitracin, teicoplanin, and fucidin have been shown to be an effective initial treatments for CDAD [54]. A significant number (20%–35%) of patients develop recurrent illness caused by the same or different *C. difficile* strains and symptoms arise several days (usually >4) to several weeks after the apparent success of the initial antibiotic therapy [55–57].

CDAD has been a recognized health problem in the United States and many industrialized countries for more than 30 years [58], but the epidemiology of the disease is changing. The prevalence and severity (case fatality rate) of CDAD continue to increase in spite of numerous discoveries concerning its epidemiology, pathogenicity, and treatment [53, 59]. This increasing trend is associated with the emergence and spread of an epidemic strain referred to as NAP1/BI (North American pulsed-field type 1, ribotype 027, restriction endonuclease analysis type BI, toxinotype III) [47, 60]. As a result, the average inhospital cost of CDAD patients is estimated to be 54% more than non-CDAD patients in the United States, adding an overall $1.1 billion to national health care costs [61]. Length of hospital stay also increases with CDAD patients and ranges from an average of 3.6 days for the total inpatient population to 16 days for surgical inpatients [62].

6. THE MICROBIAL ECOLOGY APPROACH TO AAD AND CDAD

There are few data that assess changes in the human gut microbiota during the course of AAD. The onlysequence-based, microbial ecology study to date followed a 39-year-old male throughout an amoxicillin-clavulanic acid treatment (875 and 125 mg, resp., 2 times daily for 10 days) for acute sinusitis [63]. The patient developed non-CDAD within 24 hours of the first dose and symptoms persisted until 4 days after the final dose. Stool samples were taken 12 hours after the first dose (day 0), 4 days into the 10-day regime (day 4), and at 2 weeks following the final dose (day 24). A total of 84, 74, and 84 randomly cloned 16S rRNA genes were sequenced from each sample, respectively.

At 4 days into the amoxicillin-clavulanic acid therapy, the gut microbiota of this individual was markedly shifted. Representation of the Bacteroides group went from exclusively *B. fragilis* on day 0 to almost all *B. distasonis* on day 4. There was also a dramatic outgrowth of Enterobacteriaciae (most likely *E. coli*). Lastly, all members of the Clostridial rRNA cluster XIVa and Bifidobacteria groups (32% of the all sequences on day 0) were lost or below the detection limit.

Two weeks after the last dose of antibiotic, the microbiota appeared to be recovering to day 0 composition. The *B. fragilis* and Clostridial rRNA cluster XIVa groups rebounded, while *B. distasonis* and Enterobacteriacea groups were drastically decreased or undetected. Interestingly, members of the Clostridial rRNA cluster IV group were relatively unaffected by the antibiotic treatment and were sampled at roughly even numbers on all 3 sampling days. In contrast, members of the Bifidobacteria group were lost or below detection by day 4 and remained so at day 24. These data suggest that (i) the composition of the gut bacterial community is dramatically shifted during antibiotic therapy, (ii) that resiliency to this drug's effects is group specific, and (iii) that it may require an extended period of time for the microbiota to recover to the prestressed composition, if at all. More data are needed to adequately assess the rate and extent of recovery from this and other antibiotics and to assess how variable these effects are in the human population.

The association between CDAD and perturbations of the gut microbiota is well established but poorly understood. For example, animal (hamster and mouse) and in vitro models show antagonism between conventional microbiota and *C. difficile* population growth [64]. These findings help to explain the success of bacteriotherapy for recurrent-CDAD, where the disease was resolved by rectal instillation of donor stool [65, 66]. However, the use of probiotics and synthetic mixtures of bacteria has had limited success [67] and is not currently efficacious as alternative therapies. The hope is that a better understanding of the complexity of this system during CDAD infection will lead todefined manipulations of patient microbiota that will both prevent establishment of this pathogen and treat acute disease.

To this end, Chang et al. recently applied the same approach discussed above (16S rRNA gene sequencing) to 7 patients with initial ($n = 3$) and recurrent ($n = 4$) CDAD and 3 control individuals from an outpatient clinic [68]. Species level identity based on 97% nucleotide similarity was determined for 125–184 16S rRNA genes per individual. To gain insight into the overall bacterial diversity of each patient's fecal microbiota, rarefaction curves were generated from these sequence data. Rarefaction is a method of generating idealized taxonomic "collectors curves" from community data through data resampling [69]. The shape of the rarefaction curves is then indicative of the overall complexity of the microbiota in each community, allowing comparison of the diversity of each patient's fecal microbiota.

At this level of community sampling, inferences were restricted to the most abundant members. However, and without exception, the microbiota from control and initial CDAD patients was more complex than the microbiota from recurrent CDAD patients. Furthermore, the authors were able to combine these data with those from the non-CDAD-AAD patient [63] to show a clear association between microbiota complexity and disease outcome (i.e., Controls > AAD > initial CDAD $\gg$ recurrent CDAD). This study not only provides a support for the barrier function against *C. difficile* establishment and disease, but also because the sequences represent actual organisms, these data can be used to identify potentially useful antagonistic relationships in the community.

The 16S rRNA clone library approach is useful to study interesting symbiotic associations in bacterial communities. This and other techniques may also be useful in predicting clinical outcomes based on their association with specific consortia of bacteria. To do so requires a novel conceptualization of the disease process in that one particular organism is not necessarily defined as the causative agent, but rather the entire community is involved in causing the outcome. There is little information available to generate these types of risk models, but the clinical potential in using microbial ecological inferences to guide therapies (i.e., tapering antibiotic treatments, probiotics, etc.) and prevention certainly warrants further investigation.

7. CONCLUSIONS AND FUTURE DIRECTION

Comparative studies that use microbial ecology techniques to analyze temporally sampled patients and control individuals are a promising approach to complex disease research. Traditional culture-based methods continue to be the gold standard for disease diagnostics, but this approach can only detect organisms that are easy to isolate and have simple metabolic requirements. Since the vast majority of the human gut microbiota is currently noncultivable, a nonculture-based approach may be more useful for the diagnosis and prediction of clinical outcomes [70]. Analyzing the metagenome is such an approach and can be used to identify members of complex bacterial communities based on nucleotide variability in conserved genes [70, 71]. New technologies, such as pyrosequencing, have recently become available and attain the high throughput and resolution required to make detailed community comparisons based on more than one locus. An added benefit of these technologies is that reagents and chemistries are constantly being re-engineered so that efficiency is maximized at lower cost.

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Review Article

Insights into the Roles of Gut Microbes in Obesity

Yolanda Sanz, Arlette Santacruz, and Giada De Palma

Institute of Agrochemistry and Food Technology (IATA), Spanish National Research Council (CSIC), P.O. Box 73, Burjassot, 46100 Valencia, Spain

Correspondence should be addressed to Yolanda Sanz, yolsanz@iata.csic.es

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Obesity is a major public health issue as it enhances the risk of suffering several chronic diseases of increasing prevalence. Obesity results from an imbalance between energy intake and expenditure, associated with a chronic low-grade inflammation. Gut microbes are considered to contribute to body weight regulation and related disorders by influencing metabolic and immune host functions. The gut microbiota as a whole improves the host's ability to extract and store energy from the diet leading to body weight gain, while specific commensal microbes seem to exert beneficial effects on bile salt, lipoprotein, and cholesterol metabolism. The gut microbiota and some probiotics also regulate immune functions, protecting the host form infections and chronic inflammation. In contrast, dysbiosis and endotoxaemia may be inflammatory factors responsible for developing insulin resistance and body weight gain. In the light of the link between the gut microbiota, metabolism, and immunity, the use of dietary strategies to modulate microbiota composition is likely to be effective in controlling metabolic disorders. Although so far only a few preclinical and clinical trials have demonstrated the effects of specific gut microbes and prebiotics on biological markers of these disorders, the findings indicate that advances in this field could be of value in the struggle against obesity and its associated-metabolic disorders.

1. INTRODUCTION

Obesity is a major public health concern affecting both the developed and the developing world. The obesity epidemic started to grow in US in the 1980s, with values rising from 22.9% obese adults in 1988–1994 to 30.5% in 1999–2000 [1]. In 1996, the World Health Organization (WHO) together with national Ministries of Health agreed to tackle obesity worldwide, but since then it has increased sharply, reaching values of at least 20% obese adults in most US states and European countries [2]. Obesity is detrimental to the quality of life and implies high health costs as a consequence of its associated morbidities. Overweight and obesity constitute risk factors for a number of chronic diseases including diabetes, cardiovascular diseases, nonalcoholic fatty liver disease, cancer, and other immune-related disorders such as asthma and infections [3].

Obesity results from a long-term positive imbalance between energy intake and expenditure with excessive increase in body fat. Obesity and the associated disorders are also characterized by a state of chronic, low-grade inflammation with abnormal cytokine and adipokine production [4]. Production of inflammatory immune mediators such as tumor-necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, CC-chemokine ligand 2 (CCL2 or monocyte chemotactic protein 1), and the proinflammatory adipokines leptin and resistin is usually high in these subjects, whereas production of the anti-inflammatory and insulin-sensitizing adipokine adiponectin is reduced [5]. Inflammation associated with obesity involves diverse signal transduction cascades including the nuclear factor kappa B (NF-κB)/IKKβ system and the Jun N-terminal kinase (JNK) [4, 6]. Leptin is the dominant long-term signal informing the brain of energy stores and, together with insulin, is secreted upon ingestion thus inhibiting food intake. However, human obesity is not commonly associated with leptin-deficiency but with leptin-resistance and increased levels of this adipokine. Leptin seems to exert a proinflammatory effect by inducing the production of CCL2, proinflammatory cytokines (TNF-α, IL-6, and IL-12), and also typical T helper (Th) 1-cytokines (IL-2 and IFNγ) involved in other chronic inflammatory and autoimmune disorders such as Crohn's disease [6, 7].

Although susceptibility to definitive increases in body weight is genetically determined, the environment also influences weight gain considerably. It is currently believed that macrosocial changes associated with regular intake of energy-dense foods and low-physical activity have created an obesogenic environment worldwide, constituting the cornerstone of the global obesity epidemic [8]. Traditional treatments based on calorie-restricted diets and increased physical activity have succeeded in controlling obesity to some extent [9]. Nevertheless, these strategies usually yield limited and short-lived weight reductions and most people regain some of their weight loss [3]. Neither has pharmacological therapy fully succeeded in effectively treating obesity for long-term periods and also has a number of side-effects [3, 10]. In this scenario, the identification of additional environmental factors involved in energy regulation is critical with a view to develop more efficient intervention strategies.

The human gut is populated by an array of bacterial species that coevolve with the host since birth and maintain dynamic interactions with each other throughout life. The collective genome (microbiome) of the gut microbiota contains at least 100 times as many genes as the human genome, with most serving human physiological functions [11]. The metabolic role of the gut microbiota is essential to the biochemical activity of the human body, resulting in salvage of energy, generation of absorbable compounds, and production of vitamins and other essential nutrients [12]. Thus, humans are considered superorganisms whose metabolism represents the combination of both microbial and human features [11]. The gut microbiota also regulates many aspects of innate and acquired immunity, protecting the host from pathogen invasion and chronic inflammation [13, 14]. In contrast, imbalances in the composition of gut microbiota have been associated with susceptibility to infections, immune-based disorders, and recently also with insulin resistance and body weight gain [15]. In the last decades, sound relationships between the composition of the gut microbiota and human health have been established, leading to the design of dietary strategies to favor the prevalence of beneficial bacteria to maintain a healthy status. These strategies include the administration of prebiotic oligosaccharides, which stimulate the growth and/or metabolic activity of beneficial bacteria, and also of selected bacterial strains (probiotics) in the form of functional foods and supplements [16]. Herein, the current knowledge of the relationships between the composition and functions of the gut microbiota and obesity is reviewed, including some studies intended to evaluate the effects of probiotics and prebiotics in the management of metabolic disorders.

2. GUT MICROBIOTA COMPOSITION, DIET, AND OBESITY

Obesity has been associated with increases in the relative abundance of *Firmicutes* and proportional reductions in *Bacteroidetes* by comparisons between the distal gut microbiota of genetically obese (leptin deficient *ob/ob* mice) and lean mice, as well as of that of obese and lean human subjects [17, 18]. In addition, obese human adults submitted to a hypocaloric diet (either low carbohydrate- or low fat-containing diet) showed significant increases in fecal proportions of *Bacteroidetes* paralleled to weight loss over a one-year-long intervention in a few subjects [18]; nonetheless, species diversity was reported to remain constant. Studies on the cecal microbiota of genetically obese mice and their lean littermates also related a higher proportion of *Archaea* to obesity [17]. These relationships between obesity and the gut microbiota composition were first based on DNA sequence analysis of the total distal gut microbiome of mice and humans obtained from genomic libraries or directly by pyrosequencing. Of these microbial groups, *Bacteroidetes* and *Firmicutes* constitute the dominant bacterial subdivision (>99%) among the 70 bacterial subdivisions identified in distal gut, while *Methanobrevibacterium smithii* constitutes the most prominent methanogenic archaeon among the 13 *Archaea* divisions reported to date based on 16S ribosomal DNA sequencing data [11]. More recently, diet-induced obesity in animal models has been associated with increases in the proportion of a single-uncultured clade within the *Mollicutes* class of *Firmicutes*, which was also diminished by subsequent dietary manipulations to limit weight gain, showing more specific relationships between obesity and components of the gut microbiota [19]. A study of a Chinese family, comprising 3 males and 4 females, also related the lowest *Bacteroidetes* to *Firmicutes* ratio to the overweight individual, and demonstrated a high degree of interpersonal variation in this value, ranging from 0.26 to 1.36 [20].

Differences in fecal microbiota composition were shown to predict overweight in children early in life. Children maintaining normal weight showed a greater number of bifidobacteria, while children becoming overweight showed a greater number of *Staphylococcus aureus* in feces during infancy [21]. Although the selected population group included children prone to allergy, who may show the described microbial aberrancies, the obtained results are also in accordance with the protective role attributed to breast-milk against developing obesity later in life [22], and the predominance of bifidobacteria in the gut of breastfed babies [23]. Shifts in composition of animal and human gut bacteria in response to dietary changes (a high protein/low carbohydrate or high-fat intake) have also been shown to alter microbial composition and activity in the large intestine that, in turn, could exert an impact on health [15, 24]. Obese humans submitted to a dietary intervention, based on reducing carbohydrate intake and increasing protein intake, showed reductions in populations of *Bifidobacterium*, and *Roseburia* spp. and *Eubacterium rectale* subgroups of clostridial cluster XIVa when carbohydrate intake was decreased, while no differences were detected in *Bacteroide*s or other clostridial clusters [24]. The abundance of *Roseburia* spp. and *E. rectale* group correlated well with the decline in fecal butyrate as carbohydrate intake was reduced; however, relationships to body weight were not established. Recent studies on the evolution of mammals and their gut microbes pointed out that the acquisition of a new diet is a fundamental driver for changes in gut bacterial diversity, which increases from carnivory to omnivory to herbivory [25]. Alterations in gut microbiota composition associated with

genetic or diet-induced obesity have also been shown to be reversible by oral transfer of the gut microbiota from lean mice to a germ-free recipient [19, 26] or by administration of prebiotic substrates to animal models at least over short-term periods [27]. Therefore, it seems likely that a combination of environmental (e.g. diet) and genetic factors contributes to defining unique combinations of bacteria within an individual, which could favor either an obese or lean phenotype. In this context, some authors argue that both antibiotics and probiotics have demonstrated to act as growth promoters when used in animal feeding and, therefore, could contribute to current human obesity [28]. However, while antibiotics reduce gut microbiota populations, probiotics restores their levels. Therefore, their common effect on animal weight gain can be only a consequence of their common role in preventing infections. By contrast, other scientists consider that the intentional manipulation of the composition of gut microbiota via dietary strategies is a possible tool to revert or prevent overweight and particularly metabolic-associated disorders [19, 26, 27]. Although this line of research is still in its infancy, in the following sections we summarize current evidence on the mechanisms of action of gut microbiota on metabolic and immune aspects of obesity and the consequences of its dietary manipulation by pro- and prebiotics.

3. INFLUENCE OF THE GUT MICROBIOTA ON ENERGY METABOLISM

The gut microbiota is considered a critical factor, together with lifestyle, involved in energy metabolism and obesity. Germ-free mice colonized by the distal gut microbiota of conventionally raised mice produced a remarkable increase (60%) in body fat within 10–14 days, although feed consumption was reduced compared to the control germ-free mice [29]. This process also stimulated the synthesis of leptin, and produced faster glycemia and insulinemia, paralleled to body-fat increase [29]. The microbial colonization was demonstrated to increase the host's ability to both harvest energy from the diet and store this energy in adypocites. This is thought to be achieved by diverse mechanisms including improvement of diet macronutrient utilization, generation of metabolites involved in energy balance and regulation of host gene expression. Commensal bacteria have specialized sets of hydrolyses and transporters to digest nutrients, like complex polysaccharides, that would, otherwise, be inaccessible to humans. These are the main energy sources for bacteria colonizing the large intestine and confer them a competitive advantage over transient bacteria. The microbial fermentation of undigested dietary compounds can provide approximately 10% of the daily energy supply in omnivores and up to 70% in herbivores [30]. The degradation of matrix and other dietary polysaccharides (xylans, manans, pectins, starch, and inulin) as well as host mucins lead to the generation of intermediate products (lactate, succinate, etc.) and finally short-chain fatty acids (SCFA), including butyrate, acetate, and propionate, which are almost completely absorbed along the gastrointestinal tract (Figure 1). The ability to degrade highly insoluble polymeric substrates, such as cellulose and mucin, seems to be limited to a subset of primary microbe degraders in the large intestine and requires the expression of specific substrate attachment, degradation, and uptake systems like the so-called cellulosome complex. In fact, cellulolytic species have been shown to form biofilm associations with plant surfaces in vitro, integrated by a higher fraction of *Firmicutes* and a smaller fraction of *Bacteroides,* which suggests a more prominent role of the former bacterial group in energy harvest from the diet by facilitating complex polysaccharide utilization [31]. Several clostridial clusters of *Firmicutes* are important butyrate-producing bacteria in the distal gut, such as *Roseburia, E. rectale, Eubacterium halli,* and *Anaerostipes caccae,* most of which are included in clostridial cluster XIVa [31]. Acetogenesis is another metabolic attribute of relevance to obesity identified in this clostridial cluster [31], which could partly explain the inverse relationship between *Firrmicutes* and body weight reductions in previous human intervention studies [18]. Soluble and less complex oligosaccharides such as starch and fructooligosaccharides can be utilized by other gut microbes such as *Bacteroides* and *Bifidobacterium,* which could also contribute to the generation of intermediary metabolites and finally to SCFA (Figure 1).

Although butyrate-producing bacteria would appear to be related to higher gut metabolic activity leading to overweight, butyrate is extensively utilized by enterocytes and generally regarded as a healthy metabolite [32]. The main role of butyrate is to fuel enterocytes, covering up to 70% of their energy needs and contributing to epithelial cell growth regulation and differentiation (Figure 1). Butyrate also exerts anti-inflammatory effects and seems to contribute to glucagon-like peptide 1 (GLP-1) generation, which is involved in satiety, by promoting differentiation of stem cells into positive GLP-1 L cells. Altogether, this may have beneficial effects on obese-prone subjects [32, 33]. Unlike butyrate, acetate and propionate generated in intestinal lumen can reach the blood stream and the liver through the portal vein (Figure 1). Acetate can contribute to lipid and cholesterol synthesis in the liver by activating the cytosolic acetyl S CoA synthetase 2, while propionate may inhibit lipid synthesis from acetate at least in rat hepatocytes [34]. In fact, high-propionate production through fermentation of starch or fructans has been associated with serum and liver cholesterol reduction in rats and the acetate to propionate ratio in portal blood, proposed as a possible maker of the effects of these dietary ingredients on lipid metabolism [35, 36]. Nevertheless, acetate administered at a high dose to rats and rat hepatocytes also induced AMP kinase and/or reduced SREBP-1c expression related to lipogenesis inhibition, therefore further studies should be carried out in humans to verify its positive or negative influences on lipid metabolism [37].

In addition to SCFA, hydrogen is produced by polysaccharide-degrading species and its further utilization by methanogens, acetogens and sulfate-reducing gut microbes also activates the metabolism and growth of polysaccharide-degrading bacteria (Figure 1). *Archaea*, which are the main gut methanogenic microorganisms, were also overrepresented in genetically obese mice as

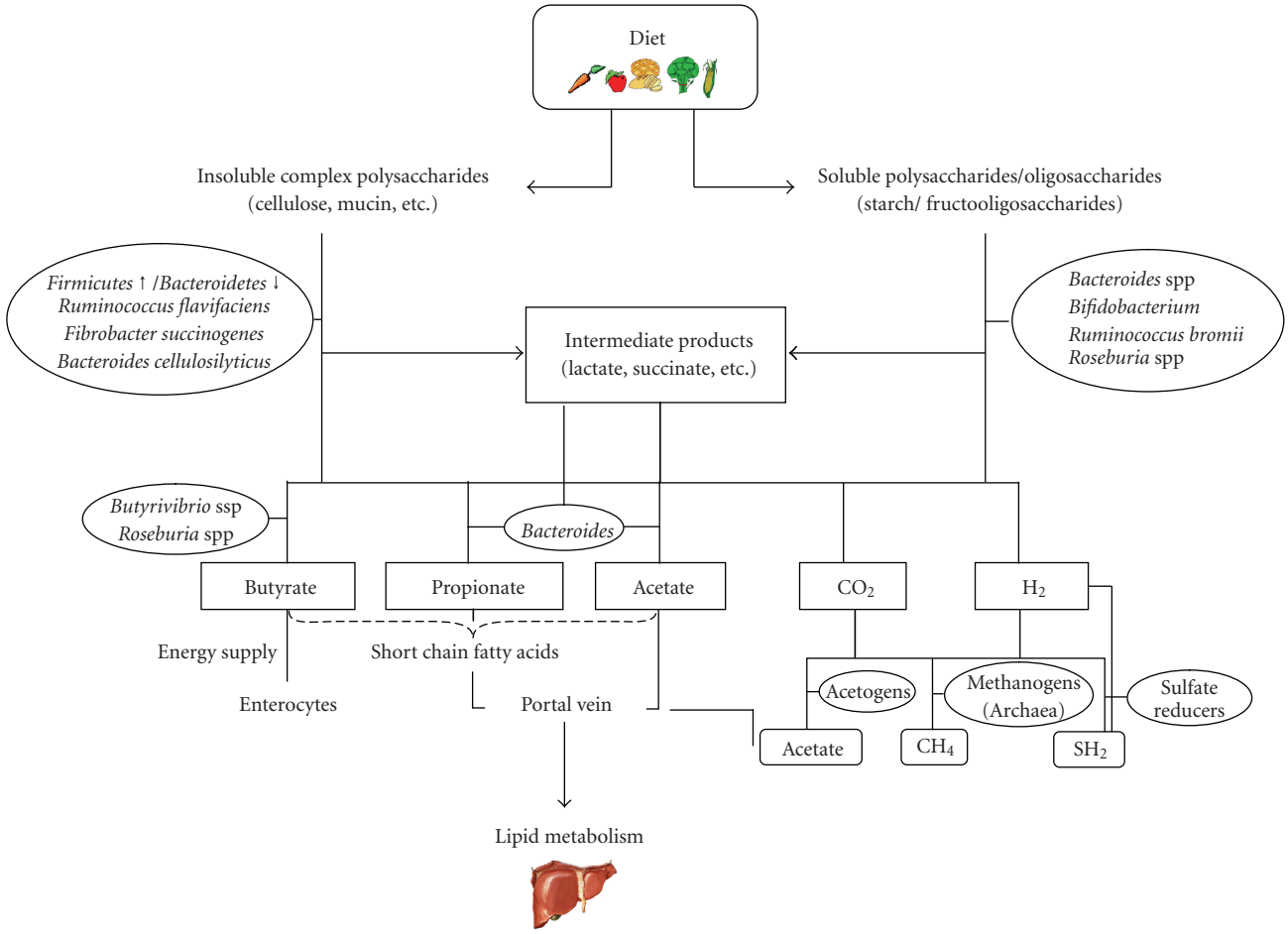

Figure 1: Schematic diagram of the main metabolic pathways of dietary poly- and oligosaccharides in the gut ecosystem.

compared to their lean littermates and were related to a greater capacity to promote adiposity when transferred to germ-free recipients [38]. *Eubacterium dolichum*, a human *Mollicute*, was also shown to favor import and processing of simple sugars in subjects under a Western-style diet, partly explaining its association and that of *Firmicutes* division with obesity [19]. In addition, cross-feeding mechanisms between components of the gut microbiota have been identified at different stages of the utilization of complex energy-rich polysaccharides. Thus, *B. adolescentis* can degrade starch, generating intermediate products (lactate and acetate) that can be utilized by butyrate-forming bacteria such as *E. hallii* to generate butyrate [39] or by other intestinal bacterial groups that convert lactate into propionate by the acrylate pathway [40]. Coinoculation of *M. smithii* and *B. thetaiotaomicron* into germ-free mice showed that *M. smithii* directs *B. thetaiotaomicron* to focus on fermentation of dietary fructans to acetate, whereas *B. thetaiotaomicron*-derived formate is used by *M. smithii* for methanogenesis. Moreover, *B. thetaiotaomicron-M. smithii* cocolonization produced a significant increase in host adiposity compared with monoassociated, or *B. thetaiotaomicron-D. piger* biassociated animals [41]. These studies emphasize the role of interactive sets of microbes, rather than the role of individual microorganism within the gut ecosystem in energy-metabolism and body weight regulation. This makes it far more complex to identify those that are critical to obesity control through dietary strategies.

The gut microbiota may also influence energy balance by modifying gene expression of host-related metabolic functions. Angiogenesis, which is primarily involved in distributing nutrients to peripheral tissues, was shown to depend on the gut microbial colonization process. Although capillary network formation was arrested in adult germ-free mice, this developmental process restarted and was completed within 10 days after colonization with a complete microbiota harvested from conventionally raised mice, or with *Bacteroides thetaiotaomicron* [42]. Commensal bacteria, such as *B. thetaiotaomicron,* have also been shown to induce expression of host monosaccharide transporters in monocolonized mice [43]. This would lead to increasing the absorption of monosaccharides and SCFA and, thereby, promote the novo synthesis of lipids in the liver. In addition, the microbial colonization of germ-free mice increased liver expression of two key enzymes involved in the de novo fatty acid biosynthetic pathways, acetyl-CoA carboxylase and fatty acid synthase, as well as the transcriptional factors ChREBP and SREBP-1, which are involved in hepatocyte

lipogenic responses to insulin and glucose [29]. Unlike colonized mice, germ-free animals were protected against the obesity that develops after consuming a Western-style, high-fat, sugar-rich diet by increasing fatty acid metabolism via two complementary mechanisms: (i) increasing levels of circulating fasting-induced adipose factor (Fiaf), which inhibits lipoprotein lipase thereby limiting fat storage in adipocytes and promoting fat oxidation in muscle; and (ii) increasing skeletal muscle and liver levels of phosphorylated AMP-activated protein kinase and its downstream targets, involved in fatty acid β oxidation [44].

Commensal gut microbiota and probiotics could also regulate serum lipids by taking part in bile acid metabolism. Bile salts are highly effective detergents that promote solubilization and absorption of dietary lipids throughout the intestine. The major bile salt modifications of microbial origin in the human gut include deconjugation, oxidation of diverse hydroxyl groups and 7 α/β-dehydroxylation [45]. Certain probiotics have been shown to decrease serum cholesterol levels by means of their bile salt hydrolytic activity [46]. Significant bile salt hydrolysis occurring in the proximal and terminal ileum reduces bile salt uptake through high-affinity transport system and lipid solubilization. This also leads to an increase in bile-acid excretion in feces and bile-acid synthesis from cholesterol [45]. For example, administration of *L. acidophilus* ATCC 43121 seemed to reduce serum cholesterol levels by bile acid deconjugation and dehydroxylation reactions during cholesterol metabolism in hypercholesterolemia-induced rats [46]. This intervention resulted in increased excretion of total fecal acid sterols and secondary bile acids (deoxycholic and lithocholic acids), and a reduction of primary bile acids (cholic and chenodeoxycholic acids). Particularly, the reduction in blood cholesterol levels was related to the increase in the insoluble bile acid, lithocholic acid. More recently, metabolomic studies have indicated that supplementation of *Lactobacillus paracasei* NCC2461 or *Lactobacillus rhamnosus* NCC4007 probiotics to germ-free mice colonized with human baby flora-induced changes in hepatic-lipid metabolism and enterohepatic recirculation of bile acids that led to a decrease in the plasma concentrations of lipoproteins VLDL and LDL, when compared to controls [47]. *Lactobacillus* supplementation also decreased fecal excretion of bile acids probably due to their accumulation in *Lactobacillus* probiotic cells. Probiotic administration also led to reductions of acetate in cecal content as well as of the hepatic acetate to propionate ratio, which was related to a reduction in serum lipids [47]. Furthermore, studies in vitro indicated that fecal commensal bacteria, but not probiotics, were able to reduce cholesterol to coprostanol and thus increasing its excretion in feces [48].

Some probiotic strains of the genus *Lactobacillus* and *Bifidobacterium* were also reported to synthesize conjugated linolenic acid (CLA) from polyunsaturated fatty acids of soy oil, which reduces serum lipids and cholesterol in liver. One example of these bacteria is *Lactobacillus rhamnosus* PL60, which is a human isolate that produces t10, c12-conjugated linoleic acid and was found to exert an antiobesity effect on diet-induced obese mice after 8 weeks of feeding. This strain reduced body weight without reducing energy intake, and caused a specific reduction of white adipose tissue without producing liver steatosis, which is a common side effect of CLA [49].

4. IMMUNE ROLE OF THE GUT MICROBIOTA AND OBESITY

Obesity is considered an inflammatory disorder, which affects both innate and adaptive immunity and favors the development of other disorders such as type-2 diabetes and cardiovascular diseases [50]. In fact, chronic activation of innate immunity is regarded as a risk factor as it favors the development of these disorders, which could also be influenced by the gut microbiota [27, 51]. The gut microbiota largely regulates innate and adaptive immunity, influencing local and systemic responses (Figure 2). The recognition of bacterial components through pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs) of innate immune cells, is considered to be the starting point of immunity, informing the immunocompetent cells to respond properly to each environmental stimulus (e.g., pathogens or harmless microbes) [13]. TLR-4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, while TLR-2 recognizes lipopeptides and lipoproteins from various pathogens, and peptidoglycan and lipoteichoic acid from Gram-positive bacteria (Figure 2) [52]. Upon ligand binding, TLR interacts with different adaptor proteins (MyD88, TIRAP/Mal, TRIF, and TRAM) activating the transcription of different downstream effector systems, such as the mitogen-activated kinases (MAPK), the NF-κB/IKKβ system, and the activator protein-1 (AP-1) with production of cytokines and diverse immune mediators [53]. Cytokines such as TNF-α, IL-1β, and IL-6 are the major proinflammatory mediators produced in response to TLR-4 stimulation by endotoxin (LPS) as well as those increased in obese and insulin-resistant patients (Figure 2) [54]. Unlike pathogenic microbes, commensal bacteria maintain a peaceful relationship with their hosts by producing a transient activation of the NF-κB cascade or its suppression by diverse mechanisms including (i) promotion of nuclear export of NF-κB subunit relA in complex with PPAR-γ [55], (ii) inhibition of IκB ubiquitination and degradation in epithelial cells [56], (iii) regulation of TLR expression and upregulation of the negative regulator Tollip protein [57], and (iv) induction of anti-inflammatory cytokines such as IL-10 [58]. TLRs and derived cytokines also play a pivotal role in linking innate and adaptive immunity through exerting action on T-cells and particularly on dedritic cells (DCs), keeping a physiological Th1/Th2 balance [13]. Th1-polarized responses characterized by overproduction of IFN-γ, IL-2, and IL-12 cytokines are associated with clearance of intracellular pathogens as well as with chronic diseases including diabetes and obesity. Most TLR-activated DCs induce differentiation of naïve CD4+ T cells into Th1 cells, while TLR2-activated DCs promote the differentiation of Th2-cells or regulatory T cells by producing high levels of anti-inflammatory cytokine IL-10 which could help to counteract the inflammatory status associated with obesity [58]. Interestingly, TLRs have been identified not only in innate and adaptive immune cells

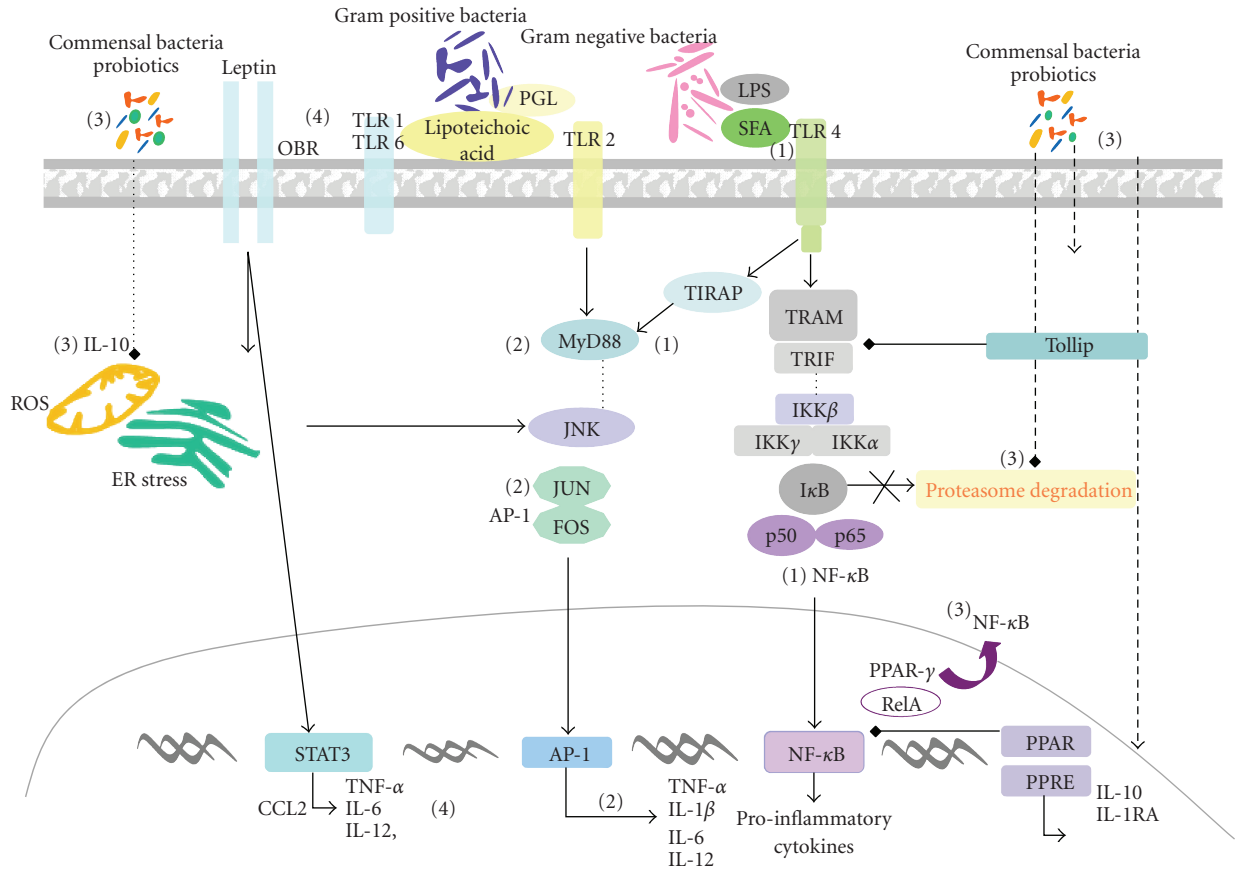

Figure 2: Schematic diagram of signaling pathways triggered by bacterial components, saturated fatty acids, and adipokines in epithelial and innate immune cells leading to either activation or negative regulation of proinflammatory pathways related to obesity and insulin resistance. (1) Lipopolysaccharide (LPS) from Gram-negative bacteria and saturated fatty acids (SFAs) is recognized by toll-like receptor (TLR) 4 activating proinflammatory pathways involving the MyD88 (myeloid differentiation primary-response protein 88)-dependent and -independent pathways that may lead to activation of nuclear factor (NF)-κB and activator protein-1 (AP-1) with production of pro-inflammatory cytokines. (2) Peptidoglycan (PGL) and lipoteichoic acid from Gram-positive bacteria are recognized by TLR-2 triggering the activation of the MyD88-dependent pathway. (3) Commensal bacteria and some probiotics may suppress activation of NF-κB cascade by (i) promotion of nuclear export of NF-κB subunit relA in complex with PPAR-γ; (ii) inhibition of IκB ubiquitination and degradation, (iii) induction of anti-inflammatory (IL10) cytokine production. (4) Leptin interacts with its receptors (OBR) activating the signal transducer and activator of transcription (STAT), and induces production of CCL2, proinflammatory cytokines, and reactive oxygen species (ROS) causing endoplasmic reticulum (ER) stress.

but also in insulin-responsive tissues such as the adipose tissue, muscle, and liver, suggesting a connection between immunity, microbial stimulation, and metabolism [59]. Diet-induced and genetically obese mice (*ob/ob* or *db/db*) showed a significant upregulation of expression of TLR-1 to -9 in adipocytes and preadipocytes along with higher cytokine production upon stimulation [60]. In particular, it is known that TLR-4 can be activated by both lipopolysaccharide (LPS) and dietary-saturated fatty acids inducing upregulation of common intracellular inflammatory pathways, such as JNK and NF-κB in adipocytes and macrophages, related to the induction of insulin resistance and increased adiposity (Figure 2) [51]. Conversely, adipocyte-specific knockdown of TLR4 prevented cytokine expression induced either by LPS or saturated fatty acids and similar effects were shown in macrophages. With some exceptions, loss-of-functional mutation in TLR-4 also prevented diet-induced obesity and insulin resistance in vivo mice models [51]. Recently, metabolic endotoxaemia, characterized by an increase in serum LPS levels, has been demonstrated to be an inflammatory factor, causative of body weight gain, insulin resistance, and diabetes in high-fat fed animal models [27, 61]. In contrast, the inhibition of the gut microbiota by antibiotic administration (norfloxacin and ampicillin) in two different mouse models of insulin resistance resulted in reduced serum LPS levels, low-grade inflammation, obesity, and type-2 diabetes, demonstrating the link between the gut microbiota and certain metabolic disorders [15]. LPS stimulation also produces a cytokine-mediated increase in plasma lipid levels by increasing the synthesis of VLDL lipoproteins in the liver and inhibiting lipoprotein lipase. In fact, mobilization of lipid stores is considered a mechanism to fuel the host's response against infections; moreover, lipoproteins also seem to help fight against infection by

binding and neutralizing LPS [62]. Therefore, common responses can be induced by "pathogenic lipid nutrients" and microorganisms mainly related to TLR-4-signaling and proinflammatory cytokine and gene transcription activation pathways. In this scenario, one can hypothesize that shifts in gut microbiota composition caused by a high-saturated fatty acid-containing diet [27], together with dietary lipids, could constitute synergic TLR signals, thus contributing to the amplification of inflammation occurring in obesity. Consequently, it has been suggested that probiotics and prebiotics with anti-inflammatory properties could be of help in the fight against obesity and associated disorders, as reported in other chronic inflammatory diseases [53]. Although few specific studies have proven such a hypothesis so far, the administration of the probiotic VSL3# was demonstrated to exert a preventive effect against type-1 diabetes in a nonobese diabetic mice model by immunomodulatory mechanisms, inducing IL-10 production in Peyer patches, and spleen and its expression in the pancreas [63]. In addition, *Lactobacillus* culture-supernatants were shown to reduce in vitro leptin production by adipocytes, thereby reducing IFN-γ production by lymphocytes and exerting an anti-inflammatory role [64]. Oral administration of a functional food product containing *L. plantarum* 299v to heavy smokers for six weeks led to a decrease in leptin, systolic blood pressure, and fibrinogen, which was attributed to the anti-inflammatory effects of this probiotic, suggesting it would be able to reduce cardiovascular risk [65]. In contrast, oral administration of *Lactobacillus acidophilus* and *Bifidobacterium longum* strains to human subjects did not influence serum leptin levels [66]. The administration of a prebiotic (oligofructose) to high-fat-diet fed mice was also shown to restore *Bifidobacterium* levels, which positively correlated with improved glucose tolerance, glucose-induced insulin secretion, and normalization of inflammatory tone by decreasing endotoxaemia in plasma and proinflammatory cytokines in adipose tissue [27].

5. CONCLUSIONS AND FUTURE CHALLENGES

Gut microbes are viewed as novel factors involved in host physiology and body weight regulation by driving a number of metabolic and immune functions. The initial association of the microbial colonization process of the germ-free intestine with body weight gain conferred a negative role to gut microbes with respect to obesity. Further, relationships established between a specific microbiota structure and a lean or obese phenotype have suggested that different microbes may influence body weight differently, and species- and stain-specific functions are being defined. In addition, endotoxaemia and dysbiosis have been identified as inflammatory factors responsible for insulin resistance and body weight, thereby returning to the concept that a healthy microbiota may be beneficial in preventing these disorders. Although the cause-effect relationships of the gut microbiota with obesity remain unclear and a limited number of in vivo trials have been done to assess the effects of specific microbial strains (commensals and probiotics), and prebiotics on metabolic disorders, the knowledge provided by these studies constitutes a breakthrough in the identification of their etiology. Further work based on systems biology coupled with "omic" technologies (metagenomics, trancriptomics, and metabolomics) will be critical to shed light on the roles of specific sets of microbes on metabolic disorders, with a view to design more efficient dietary-based strategies to reduce their risk.

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Review Article

The Human Vaginal Bacterial Biota and Bacterial Vaginosis

Sujatha Srinivasan[1] and David N. Fredricks[1,2]

[1] *Vaccine and Infectious Disease Institute, Fred Hutchinson Cancer Research Center, Seattle, WA 98109, USA*
[2] *Division of Allergy and Infectious Diseases, Department of Medicine, University of Washington, Seattle, WA 98195, USA*

Correspondence should be addressed to David N. Fredricks, dfredric@fhcrc.org

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The bacterial biota of the human vagina can have a profound impact on the health of women and their neonates. Changes in the vaginal microbiota have been associated with several adverse health outcomes including premature birth, pelvic inflammatory disease, and acquisition of HIV infection. Cultivation-independent molecular methods have provided new insights regarding bacterial diversity in this important niche, particularly in women with the common condition bacterial vaginosis (BV). PCR methods have shown that women with BV have complex communities of vaginal bacteria that include many fastidious species, particularly from the phyla Bacteroidetes and Actinobacteria. Healthy women are mostly colonized with lactobacilli such as *Lactobacillus crispatus*, *Lactobacillus jensenii*, and *Lactobacillus iners*, though a variety of other bacteria may be present. The microbiology of BV is heterogeneous. The presence of *Gardnerella vaginalis* and *Atopobium vaginae* coating the vaginal epithelium in some subjects with BV suggests that biofilms may contribute to this condition.

1. INTRODUCTION

The vagina is the Rodney Dangerfield of the human body; it gets no respect. Although frequently regarded as a mere passageway for menses, sperm, or neonates, the human vagina is a highly versatile organ that can profoundly affect the health of women and their newborn infants. The environment in the vagina can impact the probability of conception, the ability to carry a fetus to term, and the risk of acquiring sexually transmitted diseases such as HIV infection. Microbes play a critical role in determining the biochemical and inflammatory profile of the vaginal environment. Although decades of studies based on cultivation technologies have illuminated the microbiota of the human vagina, recent studies employing cultivation-independent methods have significantly increased our understanding of bacterial diversity in this important niche. This review will focus on the bacterial biota in the human vagina, with particular attention paid to studies using nucleic acid sequence-based approaches. We will highlight the changes in vaginal bacterial communities that are associated with the common condition bacterial vaginosis (BV) and will discuss the challenges to using Koch's postulates [1, 2] to assess evidence of causation for fastidious bacteria in these microbial communities. There are many important pathogens in the vaginal niche such as *Neiserria gonorrhea*, *Ureaplasma* species, *Mycoplasma genitalium*, *Streptococcus* species, *Escherichia coli*, *Chlamydia trachomatis*, and *Trichomonas vaginalis* which we will not explore in this review. Studies of fungal, viral, archaeal, and protistan diversity in the human vagina are important but will not be the focus of this review due to the paucity of published molecular surveys. Studies of the human vaginal microbiome in 2009 are in their infancy. Both metagenomic and whole bacterial genome sequencing projects are underway to help define the collection of microbial genes present in the vagina and to understand their contribution to normal host physiology and disease.

The picture that emerges from most studies of the vaginal microbiota described here is static because it is based on cross-sectional studies that assess the microbial constituents at discrete and infrequent time points. However, microbial communities in the human vagina likely undergo shifts in the representation and abundance of key species over time that are influenced by factors which may include age of the woman, hormonal fluctuations

(e.g., stage of menstrual cycle, contraception), sexual activity (e.g., types of sexual activities such as oral or anal sex followed by vaginal sex, frequency of sex, number of sex partners, and the genitourinary tract microbiota of these partners), underlying health conditions (e.g., diabetes, urinary tract infection), use of medications (e.g., intravaginal and systemic antibiotics), intravaginal washing practices and hygiene. Future studies will benefit from the use of high throughput technologies that will facilitate measuring fluctuations in the human vaginal microbiota over time in longitudinal analyses with more frequent sampling. Current data suggest that these studies will reveal a highly dynamic human vaginal ecosystem in many women.

2. THE VAGINAL MICROBIOTA: "NORMAL" VERSUS BACTERIAL VAGINOSIS

Gram stains of vaginal fluid smears from women without BV typically show Gram-positive rods, with cultures revealing a predominance of lactobacilli, particularly *Lactobacillus crispatus* and *Lactobacillus jensenii* [3–5]. Lactobacilli are believed to promote a healthy ecosystem by producing lactic acid, hydrogen peroxide, and bacteriocins that have antimicrobial properties thereby excluding pathogens from this niche [6]. *Lactobacillus iners* is an underappreciated member of the normal vaginal biota, as it does not grow on Rugosa agar that is typically used to isolate lactobacilli [3]. In contrast, women with the condition bacterial vaginosis (BV) have loss of many *Lactobacillus* species (except *L. iners*) and acquisition of a variety of anaerobic and facultative bacteria [7, 8]. Gram stains of vaginal fluid from women with BV show loss of Gram-positive rods and their replacement with Gram-negative and Gram-variable cocci and rods [9]. Cultures of vaginal fluid from subjects with BV typically yield *Gardnerella vaginalis* and a mixture of other bacteria that may include *Prevotella, Porphyromonas, Mobiluncus*, and *Mycoplasma* species. It is not known whether the primary event initiating BV is the loss of key lactobacilli or acquisition of the complex bacterial communities found in this syndrome; these may be simultaneous processes (Figure 1). It is also possible that some other factor is the primary etiological agent, and that the changes in vaginal microbiota reflect a downstream event in the pathogenesis of BV.

3. BACTERIAL VAGINOSIS

BV is the most common cause of vaginal discharge and a frequent reason for women to seek medical attention [10]. BV is highly prevalent, affecting ~10–30% of women in the United States [11], with higher rates reported in African American women and women from Sub-Saharan Africa [12–14]. Although BV is an important medical condition itself, it is associated with several more serious adverse outcomes including preterm birth [15], pelvic inflammatory disease [16], and acquisition of HIV infection [17]. Women with BV may have a malodorous vaginal discharge or local irritation, but about half of the women with diagnosable BV have no clear symptoms [18]. Some women do not report abnormal vaginal discharge, but discharge is nonetheless noted on examination by a clinician, highlighting that many women with BV are not aware of their diagnosis or consider their discharge to be within normal bounds. The high prevalence of BV and the lack of symptoms in a substantial fraction of affected women lead to the question whether BV should be considered a normal variant of the vaginal microbiota or a disease entity. For women affected by severe symptomatic BV as manifested by profuse vaginal discharge and less frequently by local burning or itching, there is little question that they have a disease. For women with laboratory evidence of BV but no symptoms, the disease designation seems inappropriate, though the condition may still impart increased risk of adverse health outcomes such as preterm birth. Antibiotics such as metronidazole and clindamycin are usually effective in treating BV in most subjects, leading to resolution of symptoms, though rates of relapse are high [19, 20]. Either systemic (usually oral) or intravaginal antibiotics can be used to treat BV.

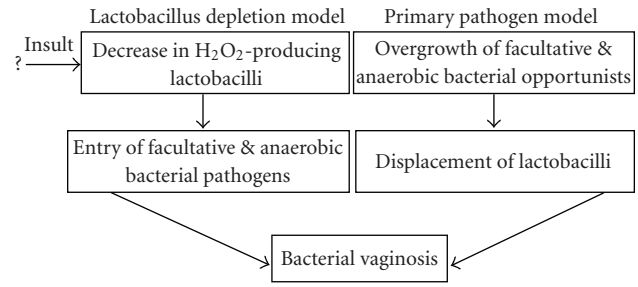


Figure 1: *Competing models for the pathogenesis of BV.* At least 2 models exist to explain the pathogenesis of BV. The lactobacillus depletion model suggests that there is a decrease in hydrogen peroxide producing lactobacilli as the primary event that allows for the overgrowth of facultative anaerobes resulting in BV. The primary pathogen model suggests that the entry of facultative anaerobes causes the displacement of lactobacilli thereby resulting in BV.

Symptomatic BV can be described as a syndrome based on the presence of a collection of clinical features without a specific etiologic agent defined. The diagnosis of BV is usually made using a series of clinical criteria collected by a clinician performing a pelvic examination, or by interpretation of vaginal fluid Gram stains. Amsel clinical criteria are usually employed for the diagnosis of BV in the clinical setting because the approach is rapid, but it does require access to a microscope [18]. At least 3 of 4 Amsel criteria must be present to establish a diagnosis of BV, including (1) elevated vaginal fluid pH > 4.5; (2) a positive "whiff test" which consists of the detection of a fishy odor upon addition of 10% potassium hydroxide to a slide containing vaginal fluid; (3) the presence of clue cells (>20%) in vaginal fluid which are shed vaginal epithelial cells coated with bacteria creating indistinct borders; (4) a homogeneous, milky vaginal discharge. Note that it is possible to have a diagnosis of BV based on Amsel clinical criteria without the presence of frank vaginal discharge. Accordingly, presuming that women without vaginal discharge do not have BV is

not valid, and studies of the "normal" vagina should ideally employ an objective method to assess for BV. Unfortunately there are numerous studies in the field that have claimed that BV-associated bacteria are part of the normal microbiota without having assessed for BV status, although self-reported vaginal discharge may have been absent. It is possible, indeed probable, that many BV-associated bacteria can be part of the normal human vaginal microbiota, but the failure to use consensus guidelines to define BV in the research setting is a recipe for scientific confusion that is completely avoidable with well-designed studies.

An alternative method for diagnosis of BV relies on analysis of Gram stains performed on vaginal fluid smears. This approach is most commonly employed in the research setting where Gram stains are used to classify subjects but is less well suited to the clinical setting because analysis of the vaginal fluid Gram stains requires a degree of expertise that is rarely available in real time when the clinician is faced with the decision whether to treat for BV. For better or for worse, the vaginal fluid Gram stain is considered the current diagnostic gold standard as it offers greater reproducibility and objectivity when compared with the Amsel's clinical criteria. For example, there can be variation between technicians in the evaluation of wet mounts for vaginal clue cells. Several scoring systems are used to classify vaginal smears. The method of Nugent et al. [9] assesses the presence and relative amounts of three bacterial morphotypes, including Gram-positive rods (lactobacilli), Gram-negative and Gram-variable rods (*Gardnerella vaginalis*, and *Bacteroides* species), and curved rods (*Mobiluncus* species). A Nugent score of 0–3 is considered normal (no BV) and is marked by the presence of Gram-positive rods, or at least no *Gardnerella vaginalis* or *Mobiluncus* morphotypes. A Nugent score of 7–10 confers the diagnosis of BV and is marked by the absence of Gram-positive rods and the presence of high concentrations of *Gardnerella* or *Mobiluncus* morphotypes. A Nugent score of 4–6 is designated intermediate flora and has Gram stain features between the two poles. Alternative scoring systems for interpretation of vaginal fluid Gram stains exist, such as that of Ison and Hay [21].

4. THE ROLE OF *GARDNERELLA VAGINALIS* IN BV

In a sentinel paper published in 1955, Herman Gardner and Charles Dukes reported the successful isolation of a novel bacterium from subjects with the syndrome nonspecific vaginitis, now known as BV. The bacterium was initially named *Haemophilus vaginalis* but was later renamed *Gardnerella vaginalis*. The authors stated, "We are prepared to present evidence that the vast majority of so-called "nonspecific" bacterial vaginitides constitute a specific infectious entity caused by a single etiological agent [22]." These investigators believed that *G. vaginalis* was the sole cause of BV and set out to fulfill Koch's postulates for disease causation in a series of clinical experiments. Pure cultures of *G. vaginalis* were inoculated into the vaginas of 13 healthy women, which resulted in the development of BV in 1 of the 13, with a corresponding rate of disease production of 7.7%. Based on these data, the investigators concluded that Koch's postulates were fulfilled, though the 92% failure rate calls this conclusion into question. The investigators went on to perform an additional experiment wherein whole vaginal fluid obtained from subjects with BV was used to inoculate the vaginas of 15 women without BV. Eleven of these 15 subjects developed BV, yielding a disease induction rate of 73%. The authors felt that these data further supported the causal role of *G. vaginalis* in BV because this bacterium was cultured from most of the induced cases. It is our interpretation of these studies that whole vaginal fluid is a much more successful inoculum for the transmission of BV than is a pure culture of *G. vaginalis*, suggesting that there are other factors besides *G. vaginalis* important in disease induction.

Other evidence suggests that *Gardnerella vaginalis* is not the sole etiological agent in BV. Koch's postulates demand that the etiological microbe should be found in every case of disease but should not be detected in subjects without disease [1] (see section on Koch's postulates). *G. vaginalis* fails this later test of specificity because it can be detected in about 30–50% of women without BV using cultivation methods and 70% of women without BV using PCR methods [23]. After more than half a century, we are still debating the role of *G. vaginalis* in BV. Although *G. vaginalis* likely plays an important role in the pathogenesis of BV, it is unlikely to be the sole instigator because it is never found as the sole bacterium in vaginal fluid from subjects with BV. Our hypothesis is that BV is a syndrome caused by communities of bacteria that include uncultivated species, precluding the formal application of Koch's postulates and necessitating new approaches for establishing causation.

5. VAGINAL MICROBIAL DIVERSITY: THE PERSPECTIVE FROM CULTIVATION

With the advent of molecular techniques used to measure bacterial diversity, it is easy to discount the contributions from studies based on cultivation because these studies may fail to detect a large number of fastidious microbes in any given niche. However, cultivation studies provide critical insights about the phenotypic characteristics of microbes that are not easily derived from molecular studies. Furthermore, cultivated microbes allow for the experimental manipulation of these organisms in the laboratory and the testing of hypotheses about pathogenesis and virulence factors. Accordingly, cultivation studies remain an important area of investigation in vaginal microbiology, despite the limitations of the approach [24]. One reason for pursuing the combined approach using cultivation and cultivation-independent methods is that some bacteria are more likely to be detected by cultivation when present in low concentrations. For example, Verhelst et al. [25] reported that of the 38 vaginal bacterial species identified from 8 subjects with and without BV, 5 were detected by cultivation alone. Novel cultivation approaches may be required to grow the many fastidious bacterial species found in the human vagina.

Prior to Burton and Reid's study in 2002 [26], almost all of our knowledge about the bacteria in the vaginal niche came from cultivation studies which involved isolating the

organisms by culture on selective or nonselective media and subsequent identification by phenotypic techniques. Just as use of a variety of broad range bacterial PCR primers helps to maximize species diversity (see section on Molecular Approaches), a number of media and growth conditions may be needed for the optimal isolation of diverse bacterial species. Relatively nonselective media such as MacConkey agar, mannitol salt agar, and tryptic soy base with 5% sheep blood agar can be useful to estimate numbers of aerobic and anaerobic bacteria in vaginal samples. Selective or semiselective media include Rogosa [27] or de Man, Rogosa and Sharpe media (MRS) for lactobacilli and the human bilayer Tween (HBT) agar for the isolation of *Gardnerella vaginalis* [28]. It should be noted that *Lactobacillus iners*, present in many subjects with and without BV, does not grow on Rogosa agar but can grow on HBT agar.

Cultivation-based approaches have identified *Gardnerella vaginalis*, anaerobic bacteria such as *Prevotella*, *Porphyromonas*, *Peptostreptococcus*, *Mobiluncus*, and *Mycoplasma* to be largely associated with the disturbed microbiota in subjects with BV. Healthy women are commonly colonized with hydrogen peroxide producing lactobacilli which are thought to inhibit the growth of the fastidious anaerobes associated with BV. Specific details of cultivation studies will not be discussed further but can be obtained from recent reviews [29, 30].

6. VAGINAL MICROBIAL DIVERSITY: THE MOLECULAR PERSPECTIVE

Cultivation-independent approaches have consistently documented the high proportion of fastidious bacteria in a variety of ecological niches [31] and these tools have recently been applied to study the vaginal ecosystem. Results from many different research groups confirm that the human vagina hosts numerous bacterial species that are either not cultivated or not easily identified using cultivation methods. These results help to augment, but do not replace, the census data generated using cultivation-based approaches. Indeed every method for characterizing the human indigenous microbiota is subject to some degree of bias. Therefore, it is our position that the most complete picture of the human microbiota will emerge from the application and synthesis of different technologies and approaches, including cultivation. We highlight both the strengths and limitations of various molecular approaches for describing the vaginal microbiota below.

The most commonly employed target for molecular identification of bacteria is the small ribosomal subunit or 16S rRNA gene. The 16S rRNA gene is useful because it is present in all bacteria and has regions of sequence conservation that can be targeted with broad range PCR primers and areas of sequence heterogeneity that can be used to identify bacteria or infer phylogenetic relationships (see [32–36]). Once the 16S rRNA gene has been sequenced from a bacterium, the variable regions can be used for species-specific PCR either in a qualitative or quantitative manner. Quantitative PCR is especially useful for rapidly identifying bacteria when an internal probe is employed and for measuring how levels of vaginal bacteria change. Nine highly variable and therefore phylogenetically rich regions of the ~1540 base pair 16S rRNA gene have been described and designated V1 to V9 [37]. The choice of primers targeting the conserved regions flanking the different variable regions can profoundly affect the diversity of bacterial species identified [38, 39].

It is theoretically possible to detect every known bacterial species if suitable broad range PCR primers or combinations of different primer pairs are employed. Current studies focus on the extraction of total genomic DNA from vaginal fluid on swabs or from cervicovaginal lavage fluid and amplification of 16S rRNA genes with primers that bind to conserved sites present in many species. The sequences obtained are aligned and compared to large databases of 16S rRNA sequences (http://greengenes.lbl.gov/ [40], http://rdp.cme.msu.edu/ [41, 42], http://www.arb-home.de/ [43] to infer phylogenetic relationships to known species. Some studies rely on the construction of clone libraries and direct sequencing of a particular number of clones [44, 45]. This approach allows for good phylogenetic resolution if a suitable portion of the 16S rRNA gene is amplified. However, this method tends to be expensive, slow, and tedious. Some investigators try to limit the sequencing of large numbers of samples by using electrophoretic fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) [46] or terminal restriction fragment length polymorphism (T-RFLP) [47]. In the case of DGGE, as the amplification products pass through the denaturing gel, their melting behavior depends primarily on the length of the product and the GC content [48]. Typically one of the primers carries a 5′-GC rich clamp, around 40 bp, which is used to detect single-base changes between close products. This clamp tends to lower the PCR amplification efficiency and can increase the presence of PCR artifacts such as heteroduplexes [48]. T-RFLP involves PCR amplification of the community DNA using primers with fluorescent tags. The resulting PCR products are digested with restriction enzymes and the fluorescent terminal restriction products are detected using a DNA sequencer. The species diversity revealed by DGGE is much less than the diversity detected by T-RFLP [49], and this likely reflects greater sensitivity of the fluorescence detection platform. Screening clones in a library by amplified ribosomal DNA restriction analysis (ARDRA) in order to limit the number of clones to be sequenced is also commonly used. ARDRA is based on the restriction digestion of 16S rRNA gene clones or amplified DNA and electrophoretic separation on high percent agarose or polyacrylamide gels [7].

An approach complementing broad range PCR is characterization of the vaginal bacterial community by using nucleic acid probes, oligonucleotides complementary to rRNA gene targets. Probes are designed using sequences generated from broad range PCR and sequencing experiments which can have a wide range of phylogenetic specificities ranging from domain to strain levels. There is also a database maintaining probes designed for many bacteria from other niches (http://www.microbial-ecology.net/probebase/) [50]. The probes are labeled with a fluorescent tag and hybridized

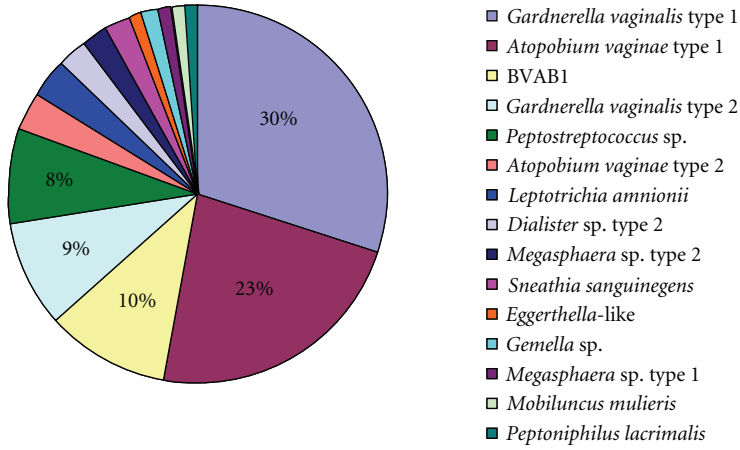
to the clinical samples. Cells are visualized using epifluorescence microscopy in a process referred to as fluorescence in situ hybridization (FISH). Data can be collected in both quantitative and qualitative modes. For example, when fluorescent probes are combined with flow cytometry, one can rapidly count and collect cells. With confocal scanning laser microscopy, one can visualize the spatial arrangement of cells in tissues or body fluids.

7. DIVERSITY STUDIES BASED ON THE 16S rRNA GENE: LIMITATIONS

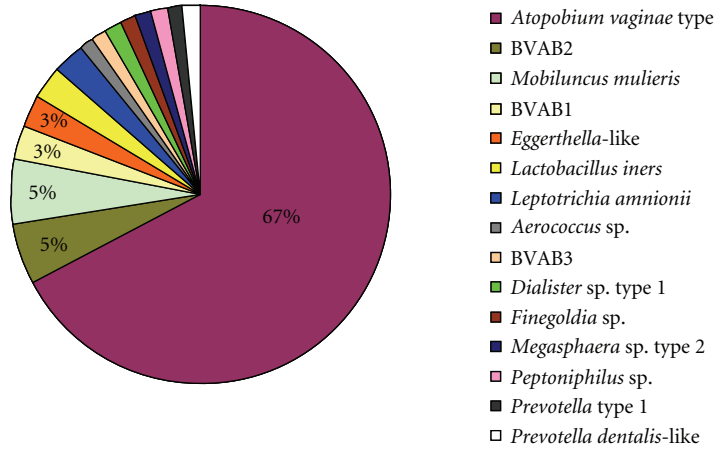
While molecular methods have many advantages over cultivation approaches for characterizing microbial diversity, there are numerous limitations [51–53]. Use of some so-called "universal primers" targeting conserved regions of the 16S rRNA gene may not detect all bacteria present in a sample due to the presence of polymorphic nucleotides at conserved positions. The primers are more accurately designated as broad range. Heterogeneity of the 16S rRNA gene within the same species can also hamper fingerprinting analysis [39]. Lowering the annealing temperature during PCR permits mismatches when using broad range primers thereby increasing the diversity of the PCR products formed, though this may also allow nonspecific amplification of DNA from human tissues. Degenerate nucleotides can help in overcoming the deficiency of broad range primers when polymorphic base positions are encountered but can lead to lower efficiency of primer binding due to exact matches of variants being diluted in the primer pool. If the primer concentrations are increased to overcome this dilution problem, then there is the potential for increased nonspecific product formation. Inosine-based primers are an alternative to degenerate primers [54] but these cannot be successfully used with *Pfu* [55], a high fidelity polymerase. One example of a commonly used broad range PCR primer targeting the 16S rRNA gene is the 27f (8f) primer at the 5′ end of the 16S rRNA gene. This primer has multiple mismatches with many Chlamydiae and Bifidobacteria, highlighting the fact that this primer may be highly inefficient in detecting bacteria in these phylogenetic groups [38]. More frequently, individual species within phylogenetic groups may have mismatches that result in reduced amplification efficiencies [30]. Frank et al. [38] evaluated the 27f (8f) primers (designated as 27f-CC and 27f-CM) that are commonly used in many broad range PCR studies and formulated a 27f primer mixture (designated as 27f-YM+3) that included three sequences not usually accounted for in many contemporary studies. These primers are better matches with bacteria in the *Chlamydiales* and *Bifidobacteriales* orders as well as bacteria in the *Borrelia* genus. Using a combination of linear amplification with the 27f formulation and quantitative PCR, they showed that the formulated primer mixture performed better at detecting *Gardnerella vaginalis* sequences even at elevated annealing temperatures (60°C) than the 27f primers typically used in the literature. Several studies have attempted to characterize the vaginal bacterial biota using the conventional 27f primer and these studies appear to underrepresent bacteria such as *G. vaginalis* which is a common member of the vaginal ecosystem [25, 56]. Although use of complex primer mixtures may increase the diversity of bacteria detected by broad range PCR, this advantage comes at a cost. When using the primer mixture, there is a slight decrease in amplification efficiency due to a reduction in primer concentrations with exact matches.

We have seen similar problems with the 27f primer in our broad range bacterial PCR studies of the vaginal niche. We amplified a region of the ribosomal RNA operon using 27f [57] modified with one degeneracy (27f-CM) and 189r [58] at an annealing temperature of 55°C. Clone library analysis on a model subject with BV revealed the absence of *Gardnerella vaginalis* (Figure 2, unpublished data). In contrast, by utilizing a different forward primer (338f) and the same reverse primer, *G. vaginalis* emerged as the dominant clone in the library (Figure 2). Moreover, as can be seen in Figure 2, use of different forward primers on the same vaginal sample results in vastly differing rank abundance plots. For example, a fastidious bacterium in the *Clostridiales* order designated BV-associated bacterium 1 (BVAB1) was detected using the 338f primer while all three novel bacteria in the *Clostridiales* order associated with BV (BVAB1, BVAB2, BVAB3) were detected with the 27f primer. The 5 most prevalent clones detected with 338f included sequences matching *G. vaginalis* type 1, *Atopobium vaginae* type 1, BVAB1, *G. vaginalis* type 2, and *Peptostreptococcus* while the most abundant clones seen with the 27f primer were *A. vaginae* type 2, BVAB2, *Mobiluncus mulieris*, BVAB1, and an *Eggerthella*-like bacterium. Using both sets of primers, we detected a total of 22 phylotypes of which 10 were represented as singleton species (detected as a single clone). When this is compared with each primer pair alone, we were able to detect only 15 phylotypes each, including 5 singletons with the 338f primer and 8 single clones with the 27f primer. We also noted that the 27f primer in combination with 189r tended to be biased to *A. vaginae*, thereby not providing representative reflections of bacterial abundance (Figure 2). However, we found that creating two clone libraries with different forward primers resulted in detection of more phylotypes, again highlighting the limitations imposed by the selection of a single primer pair. Accordingly, we suggest that using combinations of broad range primers on the same sample may maximize the diversity of species detected, though this comes at a cost of additional time and money expended.

The DNA extraction step is vital to getting a representative pool of DNA which will then be used for PCR amplification. Species bias for different extraction methods is well known [59, 60]. Presence of inhibitors in the clinical samples from blood, mucus, or vaginal products can lead to failed amplification or a reduction in the amount of product. Amplification controls are useful in tracking DNA quality wherein PCR of specific target genes such as beta-globin [23] or the 18S rRNA gene [61] can indicate if the DNA extracted from human tissues is amplifiable. Use of internal amplification controls by adding an exogenous template at known concentrations to the clinical samples can help in detection of subtle PCR inhibitors [62, 63], particularly when performing quantitative PCR analysis.



(a) Forward primer—338f



(b) Forward primer—27f

Figure 2: *Comparison of vaginal bacterial species detected by broad range 16S rRNA gene PCR using two different forward primers and the same reverse primer in one sample.* The pie charts show the percentages of clones in each library corresponding to specific bacterial 16S rRNA gene sequences obtained using broad range PCR followed by cloning and sequencing in a vaginal sample from a subject diagnosed with bacterial vaginosis. Data obtained using the 338f (a) primer shows a balanced representation of clones while the data obtained using the 27f (b) primer is skewed toward *Atopobium vaginae*. Note the absence of *Gardnerella vaginalis* clones in the clone library created with the 27f primer. BVAB denotes bacterial vaginosis associated bacterium.

Another issue with broad range PCR targeting the 16S rRNA gene is the lack of phylogenetic resolution for some bacteria, even at the species level. For example, different species within the *Enterobacteriaceae* have very similar 16S rRNA gene sequences. Other gene targets offer improved phylogenetic resolution for some species, such as the sigma factor *rpoB* present in just one copy per genome [64–67]. A downside of using *rpoB* as a marker is the dearth of sequences available when compared to the 16S rRNA gene. An alternate option is to examine the internal transcribed spacer region by ribosomal intergenic spacer analysis to distinguish closely related strains [68–70]. Here again, sequence and size heterogeneity can be critical limitations, and databases (http://egg.umh.es/rissc/) supporting this region are small in comparison to those supporting 16S rRNA gene sequences.

Correlating the number of 16S rRNA gene copies (and hence clones) to the number of bacteria is frequently not possible as different bacterial species can have varying numbers of rRNA gene operons per genome (between 1 and 15) and the exact number is unknown for most species [71–73]. Bacteria with higher rRNA operon copy numbers will be excessively represented in a clone library when compared with bacteria with lower copy numbers, thereby introducing a bias in the community analysis [74]. Moreover, different bacteria may have varying susceptibilities to lysis based on the extraction methods being used thus leading to different quantities of bacteria observed in subsequent analysis.

Similarly, false positives can impact community analysis when targeting the 16S rRNA gene using broad range primers. Low levels of bacterial DNA may be present in laboratory or PCR reagents and in DNA extraction kits. *Taq* polymerase used for PCR amplification can have contaminating 16S rRNA sequences [75, 76]. A way to monitor this problem is to include negative controls in every

run of PCR. No template PCR controls allow for detection of contaminants arising from PCR reagents and the water being used in every PCR experiment. Additionally, it is extremely useful to include extraction controls wherein sham samples are processed and extracted in the same manner as the experimental samples. These extraction controls should be subjected to PCR and analysis of products (such as cloning/sequencing) alongside samples of interest to identify any contaminants. Limiting the number of amplification cycles and using high amounts of template DNA also help in reducing amplification of low level contaminants that may have been introduced during the different steps of sample preparation. An important source of PCR contamination is from previously amplified products. This can be managed by separating pre- and post-PCR working spaces, use of aerosol filter pipette tips, and addition of uracil glycosylase to inactivate previously amplified PCR products.

The PCR amplification step itself can introduce biases such as skewed representation of a sample based on the guanosine plus cytosine (G+C) content of the bacterium [77, 78]. Bacteria with higher G+C content may result in lower throughputs when compared with bacteria with lower G+C. PCR enhancing additives such as betaine [79], dimethyl sulfoxide [80], or formamide [81] are typically used to equalize the read-through efficiencies of the different templates with varying G+C contents while the reducing environments created by β-mercaptoethanol or dithiothreitol [82] seem to provide unspecified PCR enhancing effects. PCR enhancers that are commercially available (e.g., Q-solution from Qiagen, PCR enhancer solution from Invitrogen) can be expensive and their composition is not known. Low cost in-house reagents such as a combination of betaine, dithiothreitol and dimethyl sulfoxide have been shown to improve both qualitative and quantitative outputs of PCRs [83].

PCR artifacts are a well-known limitation when using the broad range PCR approach. Incorporation of incorrect nucleotides using *Taq* polymersase may lead to errors in the sequence. Heteroduplexes may form when primers become limiting and/or there is greater template diversity [84, 85]. Use of *Pfu* polymerase which possesses 3′ to 5′ exonuclease proofreading capabilities allows for the correction of misincorporated nucleotides and hence has fewer errors when compared with *Taq* polymerase [86]. There are other high fidelity DNA polymerases that are currently available such as Vent DNA polymerase isolated from *Thermococcus litoralis* and Phusion DNA polymerase which is a *Pyrococcus*-like enzyme with a double-stranded DNA-binding domain. One recommended strategy to limit heteroduplex molecules prior to cloning is to reamplify 10-fold diluted PCR product containing mixed templates in a process referred to as "reconditioning PCR" [85]. Formation of chimeras [87, 88] needs careful monitoring and identification. Chimeric sequences are PCR artifacts that arise when two or more phylogenetically distinct sequences become combined into a single sequence when the polymerase jumps between templates during extension. Several online tools are available to detect chimeras such as Bellerophon [89], Mallard [90], or Pintail analysis [91].

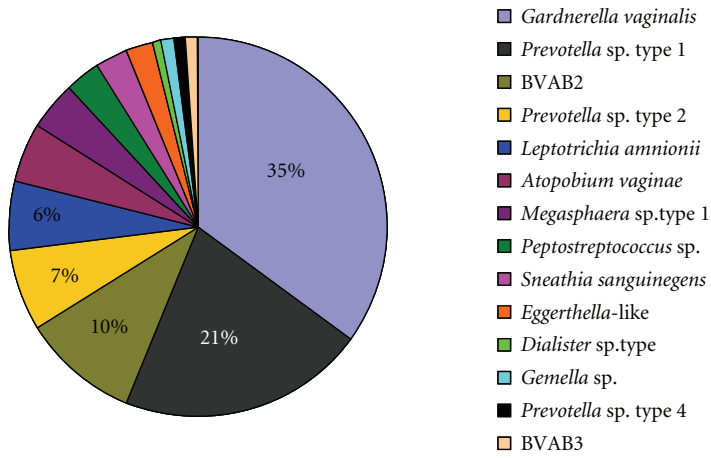
While the broad range 16S rRNA gene PCR approach provides a good census of the bacteria present in the clinical sample, no functional genomic information is obtained. Metagenomic approaches have been applied to environmental samples [92, 93] but are slow to be applied to the vaginal environment due to lack of whole genome sequence information for creation of a scaffold. There is presently an NIH-led initiative to sequence whole genomes from cultivable bacteria from the vaginal niche which will provide the necessary foundation for metagenomic studies (http://nihroadmap.nih.gov/hmp/).

8. MOLECULAR STUDIES IN THE VAGINAL NICHE: A CRITICAL EXAMINATION

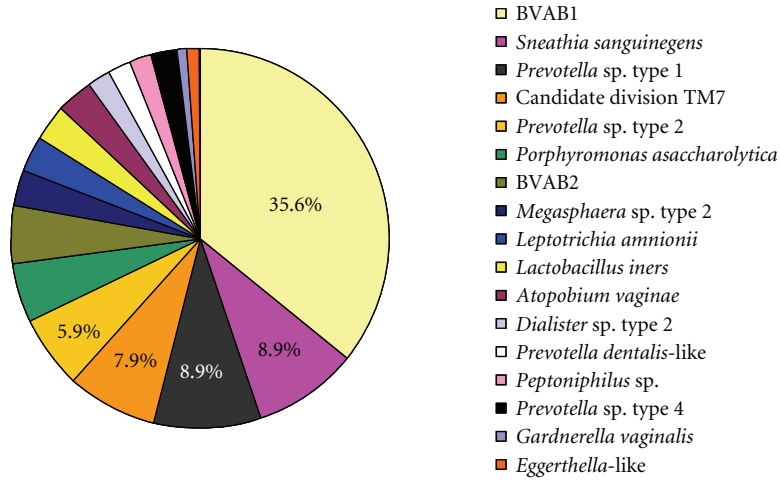
With advancing technologies and decreasing costs of sequencing, there have been many recent additions to our knowledge regarding the human vaginal microbiota. As conditions in the vagina may be transient and dependent on numerous factors, most molecular studies offer a snapshot of the vaginal microbiota under specific conditions. Moreover, with differing definitions of "normal," it can be difficult to compare the data across many studies. We present here a survey of key molecular investigations in the vaginal niche, highlighting the important contributions and the limitations of each approach.

Burton and Reid [26] were the first investigators to analyze the microbiota of the vaginal niche using broad range molecular methods. They applied a combination of broad range bacterial PCR using primers HDA-1-GC (338f with a GC clamp) and HDA-2 (515r) and DGGE to vaginal samples obtained from 20 asymptomatic postmenopausal women and used Nugent scores to distinguish between healthy and diseased states. Interestingly, 70% of the women had intermediate flora or BV as indicated by Nugent score, suggesting that women with abnormal vaginal flora were overrepresented in their study compared to the general population. Broad range PCR targeting about 200 bp of the V2-V3 variable regions of the 16S rRNA gene and DGGE analysis showed that subjects with low Nugent scores had only one to two bands, mainly derived from *Lactobacillus* species, while subjects with intermediate flora or high Nugent scores had zero to four bands representing *Gardnerella*, *Prevotella*, *Peptostreptococcus*, *Bacteroides*, *Lactobacillus*, *Streptococcus*, and *Slackia* species. The detection of *Lactobacillus iners* in subjects with normal flora by Gram stain was a novel finding. Genus specific PCR was also used to monitor the bacterial species detected by broad range PCR. The strength of this study is the utilization of both broad range and taxon-specific PCR approaches, but the DGGE method may have limited the diversity detected.

An important observation from this study [26] is that different subjects with BV had different DGGE profiles indicating heterogeneity in the composition of bacterial taxa in subjects with BV. We have observed similar results using different methods. For example, Figure 3 illustrates the differences observed in the composition and number of bacterial phylotypes in two subjects with BV. Vaginal samples were subjected to broad range 16S rRNA gene PCR using primers



(a) Subject A



(b) Subject B

Figure 3: *The microbiology of BV is heterogeneous.* Comparison of rank abundance plots from 2 subjects diagnosed with BV. The charts show the percentages of clones in each library corresponding to specific bacterial 16S rRNA gene sequences obtained using broad range PCR followed by cloning and sequencing. The most prevalent bacterial clones in Subject A include those matching *Gardnerella vaginalis*, *Prevotella* sp. type 1, BVAB2, *Prevotella* sp. type 2, and *Leptotrichia amnionii*. In contrast, the most prevalent clones in Subject B include BVAB1, *Sneathia sanguinegens*, *Prevotella* sp. type 1, candidate division TM7, and *Prevotella* sp. type 2.

338f and 1407r followed by cloning, sequencing, alignment, and phylogenetic analysis. The most prevalent phylotypes in Subject A include *Gardnerella vaginalis*, *Prevotella* sp. type 1, BVAB2, *Prevotella* sp. type 2, and *Leptotrichia amnionii*. In contrast, the most prevalent bacterial clones in Subject B include BVAB1, *Sneathia sanguinegens*, *Prevotella* sp. type 1, Candidate division TM7, and *Prevotella* sp. type 2, thereby illustrating the differences in bacterial phylotypes between two subjects with BV.

In a subsequent study from these investigators, the same primers HDA-1-GC and HDA-2 with the same PCR conditions were applied to 6 samples obtained weekly from a 51-year-old woman with recurrent BV (determined by Nugent score). Overall, 7 bacterial species were detected including *Klebsiella oxytoca, Serratia fonticola, Citrobacter freundii, Morganella morganii, Kluyvera ascorbata, Escherichia coli*, and *Staphylococcus epidermidis* [94]. None of the bacteria typically associated with the vaginal niche were detected in this study. Similarly, when the primers HDA-1-GC and HDA-2 were applied to vaginal samples from a cohort of 34 HIV-seronegative Nigerian women with BV, atypical BV-associated bacteria were detected by broad range PCR and DGGE [95]. Surprisingly, of the 34 samples, 10 had only 4 bands, 16 had 3 bands, 6 had 2 bands, and 2 had one band. If each band corresponds to a single bacterial phylotype, the bacterial diversity associated with BV in this study is substantially lower than the diversity detected in other studies and likely reflects the limits of the DGGE method employed. The dominant organism in 35% of subjects was found to be *Mycoplasma hominis.* An uncultured *Streptococcus* sp. was found in 24% of the subjects and a bacterium related to a rainbow trout intestinal bacterium was found in 26% of

subjects. The absence of several prominent BV-associated bacteria may be related to the choice of primers, although the authors used the same primers to detect *Gardnerella*, *Prevotella*, *Mobiluncus*, and *Atopobium* sp. in a previous study [26]. The different results observed in this study could also be due to differences in annealing temperatures: 56°C in the earlier study [26] and 60°C in the later study [96], or due to differences in subject populations. The primers used in these studies have a 40-mer GC clamp that has been included for DGGE analysis resulting in primers that are 60 bases long, which may contribute to inefficient amplification. Based on the data presented, the authors suggest that the bacteria associated with BV in Nigerian women are different from those bacteria associated with BV in other populations of women studied. Additional molecular studies evaluating the bacterial community associated with BV from a variety of women representing different demographic groups are required to assess the degree of heterogeneity in vaginal microbiota among women.

Zhou et al. [45] investigated the bacterial community in 5 "apparently healthy" women. The women were classified as healthy using a combination of gynecological exams and self-reported symptoms, but data on Amsel's clinical criteria or vaginal fluid Gram stains were not obtained or provided. This is a major limitation of this study as many women with BV are asymptomatic. A 920 bp fragment of the 16S rRNA gene was amplified using primers 8f, also known as 27f (actual primer sequence not specified), and 926r and the products were cloned and sequenced. Between 176 and 250 clones were sequenced from each subject resulting in 2 to 7 bacterial phylotypes per subject. Two subjects had vaginal bacterial biotas dominated by *Lactobacillus crispatus,* while *Lactobacillus iners* was detected in 3 subjects. These investigators suggest that three novel taxa were associated with the healthy vagina including *Atopobium vaginae*, a *Megasphaera* species, and a *Leptotrichia* species. However, these bacteria have been associated with BV by other investigators [7, 25, 26, 97]. As standard objective criteria were not used for the diagnosis of BV, it is difficult to draw conclusions from this study about the constituents of the normal vaginal bacterial biota.

Hyman et al. [44] surveyed the bacteria on the vaginal epithelium by broad range PCR, clone library construction, and sequencing approximately 1400 bp of the 16S rRNA gene in 20 premenopausal women who were presumably healthy. While physical exams were conducted in the clinic and the women were reported to be asymptomatic, the authors did not report data on BV status using Amsel's clinical criteria or vaginal fluid Gram stains; this is a significant limitation of the study. PCR amplification of the genomic DNA was conducted using the conventional 8f (27f-CM) and 1492r primers. The forward primer has one mismatch to *Atopobium* spp. and the reverse primer also has poor homology possibly leading to poor representation of *Atopobium* spp. in the libraries. One thousand clones were selected for each subject and sequenced from both ends using conventional sequencing. Four of the 20 subjects had only *Lactobacillus* species with very high sequence diversity indicating that these vaginal bacteria were not clonal. Nine subjects had a combination of *Lactobacillus* spp. and other bacteria including *Bifidobacterium*, *Gardnerella*, and *Atopobium*. The remaining group of 7 women did not have any lactobacilli but were colonized with mixed bacterial populations that include bacteria that have been associated with BV by other investigators. This study provides a rich resource of vaginal bacterial 16S rRNA gene sequences in GenBank, but would have been more useful if additional clinical and microbiological data had been collected to exclude women with BV or define those with the condition. These investigators detected sequences from some bacteria such as *Pseudomonas* and *Stenotrophomonas* species in clone libraries that are known PCR contaminants. It would have been helpful to describe PCR and extraction controls to prove that these bacteria are arising from the vaginal epithelium and are not spuriously detected by broad range PCR.

Verhelst et al. [25] used a combination of cultivation and molecular techniques to identify vaginal bacteria in 8 subjects of whom 3 had normal flora, 2 had intermediate flora, and 3 had BV as determined by the Gram stain method of Ison and Hay [21]. Isolates from culture studies were identified using either 16S rRNA gene sequencing or by evaluating the fingerprinting patterns of the spacer regions between transfer RNA genes. Broad range PCR with primers 10f (27f-CC, not including the first two bases of the 27f primer) and 534r was used to amplify a ~500 bp fragment of the 16S rRNA gene resulting in 854 clones from the 8 subjects. The clones were analyzed using ARDRA and clones with unique ARDRA patterns were sequenced for identification of the bacteria. A total of 38 species were identified using both approaches, of which 18 were detected by cloning only, 5 were detected by culture alone. Healthy subjects had vaginal bacterial biotas dominated by lactobacilli whereas subjects with intermediate flora or BV flora had greater bacterial diversity. *Atopobium vaginae* and several BV-associated bacteria were detected in a large number of clones generated from subjects with abnormal flora. The primers selected for broad range PCR proved to be a poor match for detecting *Gardnerella vaginalis,* which was isolated by cultivation. However, *G. vaginalis* specific PCR showed that this bacterium was associated with BV. This study underscores the importance of using a combination of approaches to attain a complete picture of vaginal bacterial diversity and the need to optimize primers for broad range PCR. The use of Gram stain analysis to evaluate BV status is commendable.

Fredricks et al. [7] evaluated the bacterial community in the vaginal niche using broad range PCR with primers 338f and 1407r amplifying a ~1000 bp fragment from the 16S rRNA gene. This approach was applied to vaginal samples from 9 subjects with BV and 8 without BV using Amsel's clinical criteria to define BV in a cross-sectional analysis. In addition, serial vaginal samples were also obtained from a limited number of subjects to study the change in bacterial composition associated with incident, cured, relapsing, and persistent BV. One hundred clones from each subject were selected and screened using ARDRA with two restriction enzymes. Inserts with unique patterns were sequenced.

Women with BV showed a high level of species diversity with a mean of 12.6 bacterial phylotypes versus women without BV who had a mean of 3.3 phylotypes per clone library. *Lactobacillus* species, particularly *Lactobacillus crispatus* and *Lactobacillus iners* were predominant in women without BV. *L. crispatus* was not detected in subjects with BV, although *L. iners* was widely prevalent. Other bacteria detected in subjects with BV included *Gardnerella vaginalis*, *Megasphaera*, *Leptotrichia*, *Dialister*, *Atopobium*, and several bacterial vaginosis associated bacteria (BVABs) from the *Clostridiales* order. Three novel bacteria from the *Clostridiales* order were highly specific indicators of BV [7]. BVAB1, BVAB2, and BVAB3 belong to the phylum Clostridium but are not closely related to any bacteria with known 16S rRNA gene sequences. A subject with incident BV had a shift from a biota dominated by lactobacilli to one with increased diversity including many putative anaerobes. A subject with cured BV had an increase in lactobacilli clones and a contraction in species diversity. A subject with relapsed BV had great diversity on day 0 with BV, followed by a contraction to predominantly *L. iners* on day 28 with cure, and then an expansion of phylogenetically rich microbiota on day 100 with relapse. A subject with persistent BV had a consistently diverse vaginal biota on days 0, 24, and 64, though there were some changes in species representation over time. A limitation of this study is that use of ARDRA to screen clones for sequencing could have underrepresented the bacterial diversity observed, as this approach tends to lump together different phylotypes with similar sequences. Moreover, only 100 clones were analyzed per library (or vaginal sample) and this limited the detection of minority species. In order to visualize the bacteria, FISH was performed on vaginal smears targeting each of the novel BVABs. BVAB1 was shown to be a thin curved rod (Figure 4); BVAB2 appears as a short, fat rod and BVAB3 is a long, lancet-shaped rod. We have performed transmission electron microscopy on a vaginal sample containing high levels of BVAB1 as determined by broad range PCR with clone library analysis, species-specific PCR, and FISH experiments. The electron micrographs show long curved bacteria with a translucent zone in the outer edge of the cells which we presume to be BVAB1 (Figure 5). This is in contrast to the larger, wider, and homogeneously electron dense cells observed in a transmission electron micrograph of *Mobiluncus curtisii* obtained from a pure culture.

We have further compiled clone library data from subjects with and without BV (Figure 6). Using broad range bacterial PCR with 16S rRNA gene primers 338f and 1407r, 1327 clones were sequenced from 13 subjects without BV (Figure 6(a)). Of the 1327 clones analyzed, 65.4% of the sequences were *Lactobacillus crispatus* and 28.8% represented *Lactobacillus iners* clones. The remaining 5.8% of clones included other bacteria such as *Gardnerella vaginalis* and other lactobacilli (Figure 6(a)). These data further validate that subjects without BV have vaginal bacterial biotas dominated by lactobacilli. In contrast, analysis of 23 clone libraries from 17 subjects with BV produced 2577 clones and demonstrated a very high degree of bacterial diversity (Figure 6(b)). Each subject with BV had an average of 14 species and the top 12 phylotypes accounted for 89%

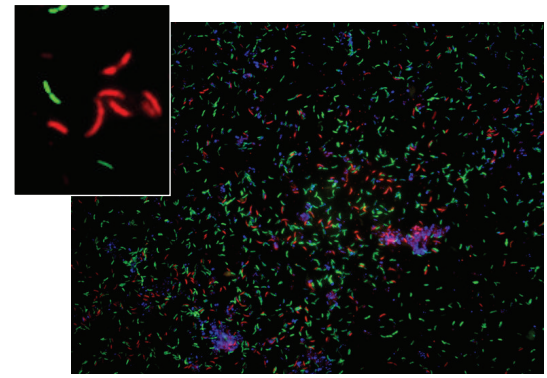
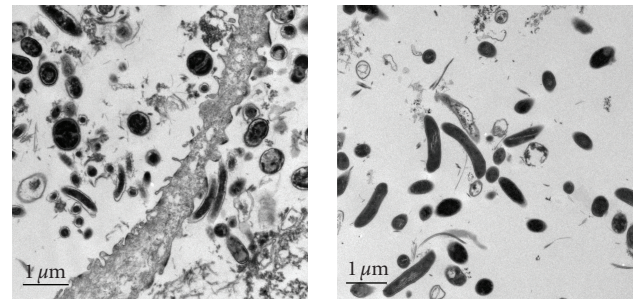


Figure 4: *Fluorescence image of vaginal fluid from a subject with BV.* Bacteria are shown hybridizing with probes targeting BVAB1 (green) and *Mobiluncus* (red) and visualized by fluorescence in situ hybridization (FISH). Other bacteria (blue) are seen with 4′,6-diamidine-2-phenylindole, dihydrochloride, (DAPI), which stains DNA. The inset shows that *Mobiluncus* (green) is larger than BVAB1 (red) but has the same curved morphology. (With permission from D. N. Fredricks, T. L. Fiedler, and J. M. Marrazzo, "Molecular identification of bacteria associated with bacterial vaginosis," *New England Journal of Medicine*, vol. 353, pp. 1899–1911, 2005.)

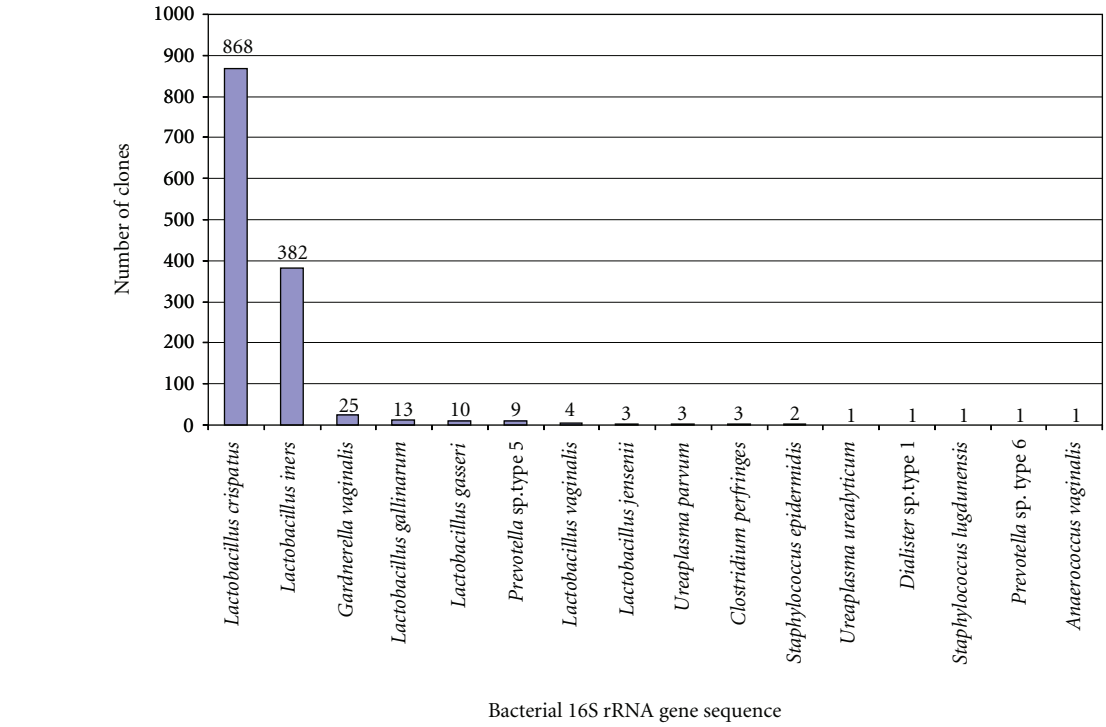


(a) (b)

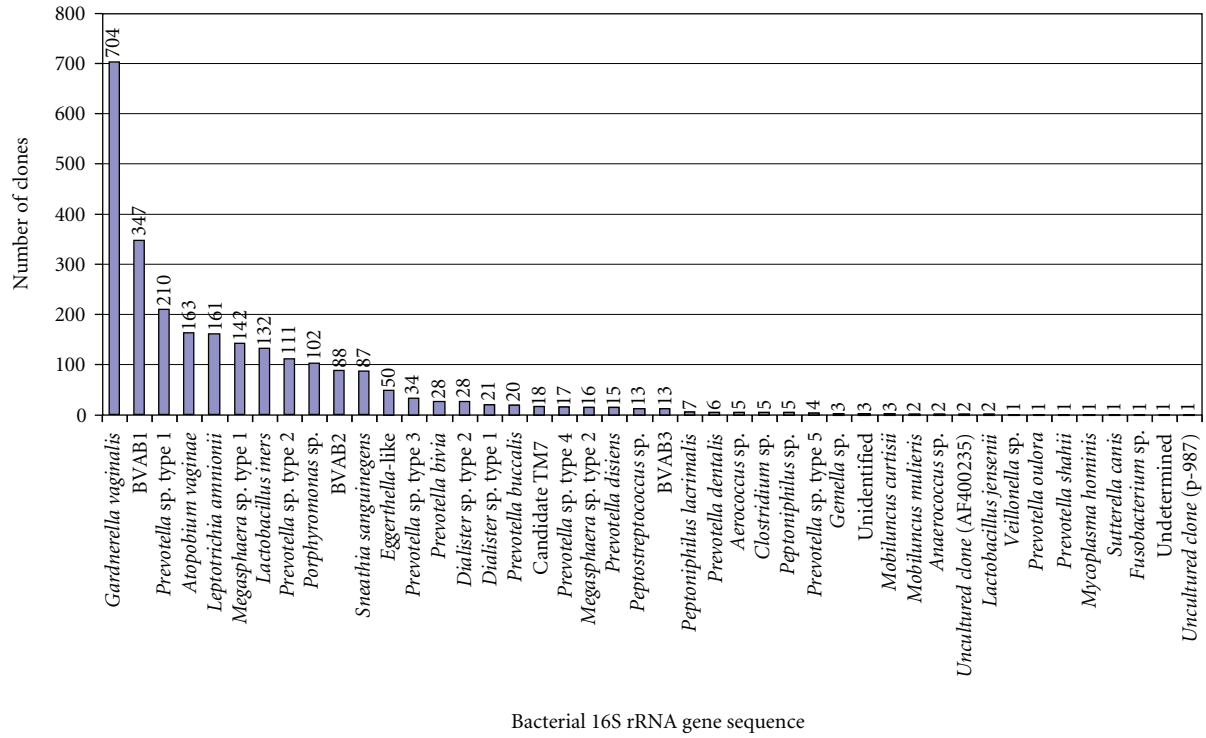
Figure 5: *Transmission electron micrographs.* (a) Electron micrograph of vaginal fluid from a woman with bacterial vaginosis and high concentrations of bacterial vaginosis associated bacterium 1 (BVAB1) shows many curved rods with an electron translucent zone at the outer edge of the cell. (b) These cells are different from the larger, wider, and more electron dense curved rods observed in a pure culture of *Mobiluncus curtisii*. Both images are at 20 000x magnification.

of clones sequenced. The remaining 11% of sequences represented 32 phylotypes. Currently, we do not appreciate the role of the "long tail" of less prevalent bacteria though it is likely that they contribute to metabolic and functional diversity in this niche. Moreover, the diversity of bacteria observed in women with BV suggests that this may be a polymicrobial syndrome.

The use of broad range bacterial PCR combined with cloning and sequencing provides a reasonable estimate of the diversity of the most abundant bacteria but is an expensive approach with low throughput. Thies et al. [98] used a combination of broad range PCR amplification of the 16S rRNA gene in combination with T-RFLP fingerprinting to



(a)



(b)

Figure 6: *Summary data of rank abundance plots depicting the bacterial species detected in clone libraries from subjects without BV (A) and with BV (B) in our studies.* Broad range PCR using primers 338f and 1407r along with clone library analysis of 1327 clones from 13 subjects without BV resulted in 16 phylotypes being detected. Similar analysis of 2577 clones from 23 clone libraries from 17 subjects with BV resulted in the detection of 44 different bacterial species. Vaginal bacterial species are indicated on the x-axis and the numbers of clones are indicated on the y-axis and above every bar. Subjects without BV have bacterial biotas dominated by lactobacilli while subjects with BV have a diverse bacterial biota. BVAB denotes bacterial vaginosis associated bacterium.

characterize the vaginal bacterial communities in vaginal swabs from 50 women with BV and 20 healthy women as determined by Nugent scoring. The authors propose that PCR combined with T-RFLP is useful to rapidly assess the most abundant bacteria and hence can be used as a tool to screen for BV. Primers for amplification included 27f (27f-CC) and 926r and were labeled at the 5′ using 6-carboxyfluorescein (6-FAM) and 4,7,2′,4′,5′,7′-hexachloro-6-carboxyfluorescein (HEX), respectively. The restriction fragment lengths were determined using an automated sequencer and the fragments were analyzed using an in-house software program. Identification of the fragments was verified by sequencing of the PCR products. A total of 23 phylotypes were detected in the samples from subjects with BV, with a mean of 6.3 phylotypes per subject (range 2–14) including *Atopobium vaginae*, *Gardnerella vaginalis*, *Megasphaera* sp., *Lactobacillus iners*, *Eggerthella* sp. and BVAB1, BVAB2, and BVAB3. Note that the species richness detected in subjects with BV in this study was less than that reported by investigators using different molecular approaches. In concordance with the results obtained in other studies [7, 44], *Mobiluncus* sp. was detected in only 2 of the 50 subjects with BV. Only lactobacilli including *Lactobacillus iners*, *Lactobacillus crispatus* group, and *Lactobacillus gasseri* group were detected in samples from subjects without BV. One of the limitations of this fingerprinting approach is the inability to distinguish between closely related species. For example, the study authors were unable to differentiate between *Mobiluncus curtisii* and *Mobiluncus mulieris* and also between the different *Prevotella* phylotypes. This resolution problem could account for the low numbers of phylotypes per subject that was observed in this study. A key strength of the study is the large number of samples processed from subjects with/without BV defined by Gram stain.

Ferris et al. [97] PCR amplified a 300 bp portion of the 16S rRNA gene with broad range primers 1055f and 1392r from vaginal samples obtained from subjects with and without BV as determined from vaginal fluid Gram stains. The DNA was subjected to DGGE and bands confirmed as *Atopobium vaginae* were identified in 12 of the 22 subjects with BV and only in 2 of the 24 control subjects. *A. vaginae* was also isolated by cultivation from 2 subjects and was shown to be metronidazole resistant. In a separate study, *A. vaginae*-specific PCR primers amplifying a 155 bp amplicon were applied to the same study cohort [99]. The specific primers further enhanced the detection of *A. vaginae* in subjects with BV while this bacterium was not detected in BV negative subjects leading to the suggestion that *A. vaginae* is highly specific for BV. PCR amplification using universal bacterial primers and T-RFLP studies also showed a correlation of *A. vaginae* to BV by Verstraelen et al. [100].

Fredricks et al. [23] used a targeted PCR approach to detect 17 key vaginal bacteria in a more sensitive fashion than is possible with broad range PCR. The PCR results were compared with the current consensus diagnostic methods for BV in order to determine if a qualitative PCR approach could be used for the molecular diagnosis of BV. Specific primers targeting various regions of the 16S rRNA gene that are specific to the bacterial species were designed. The bacteria were chosen based on clone library data previously generated [7], their apparent specificity for BV, or their novelty. All PCR products were sequenced to confirm their similarity to the intended target. The primers were applied to 264 vaginal samples obtained from 81 subjects with BV and 183 subjects without BV. Bacteria from the *Clostridiales* order, *Atopobium*, an *Eggerthella*-like bacterium, *Sneathia/Leptotrichia*, *Megasphaera* types 1 and 2, and a bacterium from the TM7 division were highly specific for BV. *Lactobacillus crispatus* was inversely associated with BV with an odds ratio of 0.02 confirming that it is largely associated with healthy vaginal flora. *Gardnerella vaginalis*, typically associated with BV, was found to have poor specificity for BV. *G. vaginalis* was found in 96% of subjects with BV but was also detected in 70% of the subjects without BV. The combination of detecting one of the *Clostridiales* bacteria (BVAB2) or *Megasphaera* type 1 produced the best sensitivity and specificity for PCR diagnosis of BV, regardless of the gold standard diagnostic criteria employed (sensitivity 99% and specificity 89%). This suggests that PCR amplification of key vaginal bacteria can indeed be used for the molecular diagnosis of BV. However, the approach used here requires electrophoresis to detect the amplification products which may not be optimal in clinical settings. A better approach would be to use quantitative PCR that offers real-time results and the ability to quantify bacteria. Levels of the bacteria may be a better indicator of disease than the presence/absence of particular species.

Some studies have investigated the utility of quantitative PCR (qPCR) as a diagnostic tool for BV. Sha et al. [101] were the first group to examine the use of qPCR for the diagnosis of BV, targeting *Gardnerella vaginalis*, *Mycoplasma hominis*, and *Lactobacillus* species using 203 samples from women with BV (Nugent score 7–10) and 203 samples from women without BV (Nugent score 0–3). Only 75 of the 203 women with BV by Nugent score were positive by Amsel criteria. Increasing levels of *G. vaginalis* and *M. hominis* and decreasing levels of lactobacilli were shown to be significantly associated with BV with a sensitivity and specificity of 83% and 78% when compared with Nugent score. The study did not evaluate women with intermediate flora.

In a subsequent study, Menard et al. [102] also investigated the association of *Gardnerella vaginalis* as well as *Atopobium vaginae* loads by quantitative PCR and assessed their utility as a diagnostic tool in 231 samples from 204 women. Nugent criteria were used to assess BV status, classifying 167 samples as normal flora, 20 samples as BV, and 44 samples as intermediate flora. They showed that the combination of the presence of *A. vaginae* at the DNA level $\geq 10^8$ copies/mL and *G. vaginalis* at $\geq 10^9$ copies/mL had a sensitivity and specificity of 95% and 99%, respectively. However, subjects with intermediate flora were excluded from this analysis. Unfortunately, the promising results from this study do not reflect how these assays would perform in a clinical setting where all women are being screened for BV, including those with intermediate flora on Nugent score. It would have been helpful to collect data on Amsel clinical criteria in these women to assess BV status using an alternative standard to determine the reliability of the

molecular approach in all women. Another limitation is the relatively small number of women with BV (20) in the study. A smaller validation cohort of 56 women was assessed, of which 7 were considered to have BV by Gram stain and 10 intermediate flora. Eleven of these 56 women had molecular criteria for BV. It is not clear if the authors are proposing to treat all women with intermediate flora for BV when they have molecular evidence of BV-associated bacteria.

Zozaya-Hinchliffe et al. [103] assessed the prevalence and abundance of uncultivated *Megasphaera*-like bacteria in the vaginal niche using quantitative PCR targeting two *Megasphaera* phylotypes in a cohort of 41 women. The subjects were diagnosed by vaginal Gram stains and Amsel's criteria. Primers specifically targeting each type were tested for cross-reactivity using vaginal clones. *Megasphaera* type 1 was detected in 76% of the subjects while *Megasphaera* type 2 was found in 52% of the subjects. Moreover, *Megasphaera* type 1 concentrations were higher in subjects with BV (up to 5 orders of magnitude) than subjects without BV, and this bacterium was significantly associated with BV ($P = .0072$), as was *Megasphaera* type 2 ($P = .0366$). Phylogenetic analysis of sequence data indicated that the *Megasphaera* phylotypes form two well-supported clades that do not match sequences originating from the rumen, gut, or oral environments, suggesting that these two phylotypes may be specific to the vaginal niche.

Current treatment strategies for BV include the administration of antibiotics either orally or topically. The use of oral metronidazole for 7 days or vaginal metronidazole for 5 days results in an improvement of symptoms in 83%–87% of women within 2 to 3 weeks [104, 105]. Similar response rates are observed with the use of vaginal clindamycin. Vaginal recolonization rates with lactobacilli are similar with both antibiotics, as defined by detection of lactobacilli on Gram stain 21–30 days after start of antibiotic treatment [106, 107]. Although there is response to antibiotics in many women, persistence or recurrence of the condition occurs in 11%–29% of women at 1 month [104, 108, 109]. Moreover, long-term recurrence rates have been shown to be greater than 70% [19, 110, 111]. Marrazzo et al. [63] investigated several risk factors for BV persistence one month after treatment, including the detection of key vaginal bacteria by species-specific PCR. Persistent BV was present in 25.8% of women at the 1-month follow-up visit as determined by Amsel's clinical criteria, also confirmed by vaginal fluid Gram stains. Taxon-specific PCRs targeting bacterial 16S rRNA genes were used to detect BVAB1, BVAB2, BVAB3, *Peptoniphilus lacrimalis*, *Megasphaera* type 2, and *Mobiluncus curtisii* at baseline and 1-month follow-up visits. Data were analyzed by presence or absence of the bacteria. *Atopobium*, *Gardnerella vaginalis*, *Megasphaera* type 1, and *Lactobacillus iners* were found in ≥96% of subjects at baseline and therefore, these bacteria were not included in the assessment of risk factors for persistence. Women with BVAB1, BVAB2, or BVAB3 at baseline were shown to have a 2–8-fold increased risk of persistent BV. Likewise, presence of *P. lacrimalis* or *Megasphaera* type 2 at baseline imparted a >3-fold increased risk of persistent BV. Other risk factors such as sexual behaviors commonly linked with persistence were also examined but were not associated with persistent BV in this study. A limitation of this approach is that the persistence data was based on qualitative detection of bacteria rather than quantitative analyses. Quantitative PCR would help determine if the bacterial levels remain unchanged during antibiotic treatment (antibiotic resistance), or if the levels decline but bacteria are not eradicated, allowing for a future relapse. Another limitation of this study is the focus on women who have sex with women. It is not clear if the same patterns will hold in heterosexual women with BV.

Oakley et al. [112] performed a systematic analysis of bacterial diversity in women with and without defined BV, incorporating data from Genbank that included publicly available 16S rRNA gene sequence data obtained from the vaginal niche. A total of 969 sequences were aligned and assigned taxonomic classifications using the Greengenes 16S rRNA gene database [113]. The sequences were further analyzed based on self-similarities rather than in comparison with an external database and classified into operational taxonomic units (OTUs) using the DOTUR software package [114] at a 97% sequence similarity cutoff, which is commonly used for species definition [115]. Indeed, subjects with BV had a much greater diversity of bacteria; at the 97% cutoff, women with BV had three times the number of OTUs (15 OTUs) when compared with subjects without BV (5 OTUs). An interesting observation made in this study was that even though there was quite a bit of variability in the bacterial species between different subjects with BV, at the phylum level, the presence of bacteria from Bacteroidetes and Actinobacteria was strongly associated with BV. The authors point out that studies assessing bacterial diversity in the vaginal niche might be underestimating the true diversity by labeling bacteria with the NCBI-based designations that lump bacteria with known species. For example, sequences classified as *Prevotella* using the NCBI classification scheme of the Greengenes classification tool actually represented 21 OTUs based on the 97% cutoff using the DOTUR analytical tool, revealing an unexpectedly high number of vaginal phylotypes or species in this genus. These different vaginal phylotypes may have different functional, metabolic, and inflammatory properties. A limitation of the study by Oakley et al. [112] is that the Greengenes NCBI classification tool used may assign different identities to the same sequence simply based on sequence length. For instance, two sequences of 100% identity but different lengths can be designated as either *Gardnerella* or *Bifidobacterium*. Similarly, two identical *Atopobium* sequences different only in sequence length can either be *Atopobium* or *Olsenella*. Sequences classified as *Bifidobacterium* in the NCBI classification scheme of the Greengenes database were classified as *Gardnerella* in the RDP database. This discrepancy highlights the larger problem of defining bacterial nomenclature, which is a continuing challenge for microbial ecologists. One way of addressing this problem is to create a database of reference sequences to which all new sequences from the same niche are submitted. This would also allow rigorous tracking of novel sequences. As we develop greater understanding of the ecology of the vaginal ecosystem, we hope that all researchers will be

able to use the same taxonomic nomenclature to facilitate comparisons across studies. For example, there is a human oral microbiome database that provides cross-referenced taxonomic and genomic information for approximately 600 species (http://www.homd.org/) [116].

Zhou et al. [56] studied vaginal bacterial communities in Caucasian and African American women in the United States . They applied T-RFLP analysis to 144 women ranging equally in ages and racial groups from various locations in the US. The subjects were classified as healthy based on examinations by medical personnel, but again BV status was not reported using either Amsel clinical criteria or Gram stain assessment of vaginal fluid. Restriction fragment pattern analysis resulted in the identification of 12 bacterial communities present in at least 2 women, and 8 communities present in single subjects. Using broad range 16S rRNA gene PCR primers 8f (27f-CC) and RD1r [117], 57 clone libraries were analyzed and ~6000 clones were sequenced. Phylogenetic analysis of the 16S rRNA gene sequences obtained led to the classification of the bacterial biota into 8 "supergroups." Five of the 8 supergroups were dominated by lactobacilli, representing 80% of the women sampled. Supergroup III, accounting for 16.5% of women sampled, had low levels of lactobacilli and a diversity of bacteria that multiple other groups have associated with BV, such as *Atopobium vaginae*, bacteria from the *Clostridiales* order, *Megasphaera*, *Dialister*, *Anaerococcus*, *Finegoldia*, *Peptostreptcoccus*, and *Eubacterium*. Since objective criteria were not used to assess for BV status (or were not reported), it is unclear if these subjects had BV or whether BV-associated bacteria colonized women without BV in this study. The study authors analyzed whether the bacterial community "supergroups" were associated specifically with race. Statistical analysis showed that supergroups III and VIII (containing a single clade of *Lachnospiraceae*) were found more often in African American women. Vaginal bacterial communities not dominated by lactobacilli were found in 33% of African American women and 7% of Caucasian women. It is known that African American women have a higher rate of BV than Caucasian women [11]. The racial differences in vaginal microbiota of "healthy" women noted in this study may simply reflect the failure to assess for BV. A substantial fraction of women with BV are asymptomatic, therefore assessing for BV status based on self-report of symptoms, as done in this study, is unreliable. Nevertheless, the fact that African American women have a higher prevalence of BV and therefore tend to have more diverse vaginal bacterial communities begs for an explanation. Strengths of the paper include the large number of samples processed, the use of T-RFLP to screen for community types, and rigorous statistical analyses applied. Limitations are the lack of objective diagnostic criteria for BV and use of a 27f primer for broad range analysis with poor homology to some vaginal bacteria that may account for the almost negligible abundance of *Gardnerella vaginalis* detected. The meaning of bacterial community "supergroups" is diminished when key members of the vaginal bacterial community are underrepresented, though this is a problem that is shared by all studies using broad range PCR to some degree.

One study assessed the vaginal microbiota from 16 women without BV (assessed by Nugent score <4), using a PCR-based approach targeting the chaperonin-60 gene (*cpn*60) [118]. Chaperonin-60 is present in all bacteria and is required for the folding and assembly of proteins and protein complexes. Most subjects were colonized largely with lactobacilli including *Lactobacillus crispatus, Lactobacillus gasseri, Lactobacillus jensenii*, and *Lactobacillus iners*. Other sequences identified included those with similarity to *Gardnerella vaginalis*, *Porphyromonas* spp., *Megasphaera* spp, and *Chlamydophila psittaci*. This is the only study that has examined the diversity of bacteria in the vaginal niche using a different target gene. This study provides a nice corroboration of results from studies using the 16S rRNA gene as a target, wherein lactobacilli have been shown to dominate the bacterial biota in subjects without BV. The detection of *C. psittaci* as part of the normal vaginal flora is interesting and rather surprising since this Chlamydia species is considered a respiratory and zoonotic pathogen and has not been previously detected in the human vagina, though it has been detected in the ovine vagina. Using a different target gene offers a different perspective on the constituents of a microbial community. However, the limited database of *cpn60* gene sequences may hinder accurate bacterial identification and the generation of phylogenetic inferences.

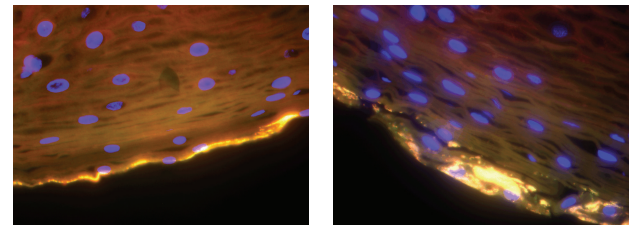
9. PYROSEQUENCING: A HIGH THROUGHPUT SEQUENCING APPROACH

While conventional sequencing techniques have provided us with a framework, the true extent of bacterial diversity in the vaginal niche is poorly understood. Analysis of the sequence data from 100 even 1000 clones results in a library with a long tail of many phylotypes detected as singlet clones when the data is represented in rank abundance plots. Based on culture techniques, it is estimated that the density of vaginal bacteria per gram of vaginal fluid ranges up to 10^8 colony forming units [119]. If a subject has 10^8 bacteria/gm of vaginal fluid and 100 clones are characterized, bacteria present at 10^6 CFU or below are less likely to be included in the analysis. Moreover, classical clone library analysis tends to provide less emphasis to the long tail of minority species [51]. In fact, the census of bacteria present at low concentrations may provide important details about genetic and functional diversity in this niche [51, 120, 121]. This is especially relevant in a syndrome such as BV where we still do not understand the pathogenesis of infection.

An alternate approach for obtaining large numbers of sequences is by using pyrosequencing technology. Pyrosequencing is a "sequencing by synthesis" method which involves taking a single strand of DNA to be sequenced and sequencing the complementary strand enzymatically while monitoring the photons generated with the addition of each base [122]. The technology was applied on a small scale level to identify isolates by analyzing the signature sequences of the V1 and V3 regions of the 16S rRNA gene in 96 well plates [123, 124]. A disadvantage of the early approach was the very short read lengths obtained (25 to 100 nucleotides long) limiting accurate phylogenetic classification.

Currently, pyrosequencing technology has been further developed and it is now possible to achieve longer reads of 250 to 300 bps in a throughput of 400 000 reads per 7.5 hour run which can generate over 100 million bases (Genome Sequencer FLX System—454 Life Sciences). The extracted DNA from the vaginal sample can be amplified using fusion broad range primers (modified with adaptor sequences) targeting the variable regions of the 16S rRNA gene. The PCR products with the adaptor sequence are attached to microscopic capture beads. Emulsion-based clonal amplification (emPCR) can create several copies of the target 16S rRNA gene sequence per bead without the need for cloning the sequences into bacteria. The beads are then transferred to a picotitre plate for sequencing. Pyrosequencing technology has been used for microbial community analysis in a variety of environments [125–130].

Sundquist et al. examined the bacterial biota in vaginal samples from 6 pregnant women in all three trimesters of pregnancy using broad range bacterial PCR with deep pyrosequencing [129]. Most of the bacterial 16S rRNA gene was amplified by PCR and portions of the gene were then subjected to pyrosequencing. A total of 100 000 to 200 000 sequence reads of about 100 bp average length were obtained for each of the 6 samples. Each read was processed using the BLAT tool, a BLAST-like alignment tool [131], and a database of bacterial sequences obtained from RDP and archaeal sequences from *prokMSA*. Two major roadblocks were faced by the study investigators. First, the short read lengths made it challenging to assign phylogeny to the sequence reads. For example, while 90% of the reads were identified to the domain level, less than 10% were identified to the species level. Only about 50% of sequences were unambiguously assigned to the class level, and this was likely due to the amplification of both conserved and variable regions of the 16S rRNA gene, limiting phylogenetic resolution. The authors also performed simulation calculations and showed that increasing sequence read lengths up to 800 bp had significant impacts on phylogenetic assignments. Current 454 technology allows a read length of 250 bp with 400 bp reads on the horizon or in place at the time of this review. The second challenge encountered by the investigators was the lack of sequences in public databases resulting in many bacteria being classified as "unknown." This problem will improve with time as more sequences are added to the databases. In accordance with data obtained from conventional cloning and sequencing experiments, the Sundquist study showed that subjects were largely colonized with lactobacilli, with a variety of other bacteria at lower concentrations including some such as *Comamonas*. In our hands, *Comamonas* spp. are common PCR contaminants that are typically present in water samples. As the study did not report results from negative controls such as sham DNA extractions with PCR and subsequent pyrosequencing, it is difficult to evaluate if *Comamonas* is indeed a part of the vaginal bacterial biota. It is imperative to conduct appropriate negative controls especially for pyrosequencing studies as the technique involves deep sequencing and can therefore easily pick up contaminating sequences even at low concentrations.



(a) (b)

Figure 7: *Vaginal biopsy from a subject with BV.* A *Gardnerella vaginalis* biofilm (yellow) is detected at the edge of the vaginal epithelium (bottom) by fluorescence in situ hybridization (FISH). The yellow color is the result of using a combination of probes targeting *G. vaginalis* (Red), all bacteria (Eub338, green), and 4′,6-diamidine-2-phenylindole, dihydrochloride (DAPI, blue) which stains DNA. Note human cell nuclei in blue. The image on the right shows a vaginal epithelial cell with a cluster of *G. vaginalis* breaking off the epithelium and likely forming a clue cell.

10. BACTERIAL VAGINOSIS: A BIOFILM SYNDROME?

Biofilms are strongly associated with human infections and up to 65% of infections treated by physicians in the developed world have been attributed to biofilms [132, 133]. There is emerging new evidence that biofilms are associated with BV [134] and it has been suggested that this biofilm may be critical in pathogenesis. Swidsinski et al. [134] demonstated the presence of adherent bacterial biofilms in 90% of subjects with BV while only 10% of subjects without BV exhibited a similar biofilm. Adherent biofilms were defined as lawns of bacteria that were tightly attached to the vaginal epithelial surface and contained specific bacterial groups. Biopsies collected from women with and without BV were sectioned and fixed for FISH and hybridized with a variety of bacterial rRNA-targeted probes. Typically, subjects with BV had an adherent biofilm that was primarily composed with 3 bacterial groups: *Gardnerella vaginalis* was present in 60 to 90% of the biofilm mass, *Atopobium* accounted for 1 to 40% of the biofilm mass, and lactobacilli were present between 1 to 5% in only 20% of the biopsy samples. Subjects without BV either had no biofilms with only a few lactobacilli scattered sporadically or had a loose bacterial biofilm which did not have any particular structure and was mainly composed of *Lactobacillus* species.

Preliminary data from our laboratory also indicates the presence of adherent biofilms in subjects with BV (Figure 7). Biopsies obtained from women with and without BV were fixed in alcoholic formalin, sectioned and examined using FISH with a suite of bacterial rRNA-targeted probes and 4′,6-diamidino-2-phenylindole (DAPI), a DNA binding fluorescent stain. Our data also suggests the presence of a *G. vaginalis* biofilm in women with BV (Figure 7) while subjects without BV did not have a biofilm but had scattered *Lactobacillus* species.

More recently, Swidsinski et al. evaluated the effect of oral metronidazole on the BV biofilm [135]. A cohort of 18 subjects with BV, diagnosed by Gram stains and Amsel criteria, were treated with oral metronidazole for 1 week.

Subsequently, follow up assessments were conducted at 1-week intervals for 5 weeks, with 3 subjects representing each point in time. Vaginal biopsies were examined using FISH probes targeting all bacteria or specific bacteria such as *Gardnerella vaginalis*, *Atopobium*, *Lactobacillus* spp., *Bacteroides/Prevotella*, and *Enterobacteriaceae*. Although, all subjects studied were considered cured of BV at the end of the antibiotic therapy, vaginal biopsies revealed a persistent biofilm. During antibiotic therapy, the biofilm could be visualized with DAPI (a DNA stain) but had poor uptake of FISH probes targeting rRNA suggesting that the bacteria were not actively metabolizing. However, at the end of 5 weeks, an actively metabolizing adherent bacterial biofilm was detected which primarily consisted of *G. vaginalis* and *Atopobium* sp. [135]. Clinically, recurrence of BV was not documented due to the limited follow-up time in the study. Important limitations of this study, also noted by the authors, include the small sample size and lack of baseline data. Furthermore, the dataset was treated as a longitudinal cohort but each time point represented a group of 3 different subjects. Despite these limitations, this study represents a novel approach to understanding the pathogenesis of BV.

Bacteria in biofilms respond differently to antibiotic treatment when compared with their planktonic counterparts [132, 136–138], and antibiotic resistance is postulated as one of the reasons for persistent and recurrent BV. A study has shown that planktonic *Gardnerella vaginalis* are more sensitive to hydrogen peroxide (5-fold) and lactic acid (4–8-fold) than *G. vaginalis* biofilm bacteria, highlighting the physiological differences that exist in the same organism under different growth conditions [139]. Several explanations are provided in the literature for the tolerance to antimicrobials by biofilm bacteria including reduced penetration of the antimicrobials within the biofilm and alterations in the stress physiology of the biofilm bacteria (reviewed in [140]). In order to circumvent issues of antibiotic resistance in bacterial biofilms, one study has used a probiotic approach to attempt clearance of the *G. vaginalis* biofilm [141]. *G. vaginalis* biofilms grown in vitro were displaced with *Lactobacillus reuteri* RC-14 and to a limited extent with *Lactobacillus iners*, commonly found in the vaginal niche. Future studies evaluating the structure and composition of biofilms in BV will become critical in understanding the pathogenesis of this common condition.

11. BEYOND KOCH'S POSTULATES: MOLECULAR GUIDELINES FOR CAUSATION

Robert Koch and his students elaborated a series of postulates to determine which microbes caused diseases and which microbes were colonizers without a direct etiological role (Table 1, Koch's postulates). The birth of modern microbiology in the latter half of the 19th century necessitated a system to gauge evidence of causation concordant with the discovery of numerous human and animal associated microbes through laboratory propagation. These guidelines, later called Koch's postulates, are elaborated in Koch's paper "On the Etiology of Tuberculosis" where he beautifully lays out the foundation for his thinking. Robert Koch was a prescient giant of microbiology whose thinking has served us well through more than a century of use. However, the power of Koch's postulates arises not from their rigid application, but from the spirit of critical judgment that they foster.

Table 1: Koch's postulates [1].

The etiologic microbe should be found in every case of the disease
The etiologic microbe should not be found in subjects without disease (specificity)
The etiologic microbe should be isolated in pure culture on lifeless media and be capable of causing the characteristic disease anew upon inoculation in a susceptible host
The etiologic microbe should be reisolated from the experimentally inoculated host.

Table 2: Limitations of Koch's postulates.

Ignore the contribution of host, vector, and environment to disease susceptibility/response
Colonization state (e.g., +PPD skin test for tuberculosis in the absence of disease) violates Koch's second postulate
Many pathogens cannot be propagated on lifeless (cell-free) medium in the lab; these pathogens cannot fulfill Koch's third postulate
Viruses, parasites, uncultivated bacteria may not grow in pure culture
Host range restriction of pathogens
Do not consider the possibility of disease produced by a microbial community rather than a single pathogen
Not completely specific

The esteemed researcher Edward Rosenow provided evidence that a streptococcus was the cause of poliomyelitis by fulfilling Koch's postulates [142–144], only to have this theory overturned with the discovery of poliovirus decades later. The lack of specificity demonstrated by Rosenow's false attribution of causation to streptococci in the case of polio highlights only one of many possible limitations of Koch's postulates that have emerged after more than a century of reflection (Table 2). These limitations do not seriously undermine the generally highly specific ability of Koch's postulates to identify true pathogens. If a pathogen fulfills Koch's postulates then it is most likely the cause of the disease, though these results need to be reproducible and consistent. In the case of *Gardnerella vaginalis* and BV, the ability of a pure culture of *G. vaginalis* to produce BV in 1 of 13 inoculated subjects is not a very compelling argument for causation without a better explanation for the 92% failure rate (see Section 4). Taken to its logical extreme, the successful induction of AIDS in 1 of 1000 subjects inoculated with *Mycoplasma* would also not "fulfill" Koch's third postulate for the role of *Mycoplasma* in AIDS in any meaningful or rigorous fashion. Nevertheless, the experimental reproduction of disease using pure cultures

of microbes is the most powerful single approach for establishing a causal connection between a microbe and a disease. On the other hand, the failure to fulfill Koch's third postulate does not mean that a microbe is not the cause of a disease. Koch's postulates have excellent specificity for causation, but poor sensitivity. For example, many microbes have not been successfully propagated in pure culture in the laboratory; these microbes cannot fulfill Koch's postulates as originally defined. The historical evolution in thinking about causation and Koch's postulates is described elsewhere [1, 2].

12. A MOLECULAR VERSION OF KOCH'S POSTULATES

A major limitation of Koch's postulates is the failure to account for the possibility that uncultivated microbes play a role in disease. The use of molecular methods to characterize microbial diversity in many niches has revealed that cultivated species constitute a minority of microbes in many ecosystems, including in the human body. Many potential pathogens can be readily detected using molecular methods such as PCR. Koch's postulates can be directly translated into molecular versions, as follows.

(1) The etiologic microbe or its nucleic acid sequences should be found in every case of disease. This implies that the microbe (or its products) is a sensitive indicator of disease.

(2) The etiologic microbe or its nucleic acid sequences should not be found in subjects without disease. This implies that the microbe is a specific indicator of disease.

(3) Experimental manipulation of infection through factors such as antimicrobial agents or induction of immune responses should demonstrate that changes in levels of an etiologic microbe correlate with disease state in the host.

13. DISEASE BY MICROBIAL COMMUNITY

There are some disease syndromes that may be caused by consortia of microbes rather than single pathogens. Examples of these polymicrobial syndromes are gingivitis, periodontitis, and BV. Proving that a single cultivated or uncultivated microbe is the cause of a disease can be challenging. Proving that a microbial consortium is the cause of a disease is even more daunting.

Microbes probably exist in communities in order to take advantage of syntrophic relationships wherein the metabolic end product of one species is the energy source for a second species. If critical members of the community are lost, then the metabolic networks collapse and all members of the community may suffer. However, functional redundancy among microbes may mean that bacterium A is not necessary for community health as long as bacterium B is present with its overlapping metabolic capacity. What does this mean if bacteria A and B are part of a pathogenic community? It means that neither bacterium will be deemed necessary for disease, because subjects may have disease when lacking bacterium A or B, though subjects will not have disease if lacking both bacteria. Bacteria A and B are considered sufficient when part of the larger community, but not individually necessary for establishing the community and producing condition. To address this issue, we will need to assess not only the species composition of pathogenic microbial communities, but also the metabolic capabilities and interdependencies of these communities. Studies of the human microbiome will be vital in filling this knowledge gap.

14. CONCLUSIONS

In the last two decades, there has been a dramatic increase in our understanding of the bacterial biota in a variety of ecological environments using cultivation-independent molecular methods. These methods have recently been applied to the human vaginal microbial ecosystem, adding substantial data on bacterial diversity in this niche. Subjects without BV have bacterial biotas that are less complex and are dominated by *Lactobacillus* species. Subjects with BV have loss of *Lactobacillus crispatus* and acquisition of more complex vaginal bacterial communities that include many heretofore-uncultivated species. Data emerging from molecular investigations suggest that it is possible to develop a PCR-based strategy for the diagnosis for BV. BV may be an example of a condition produced by a pathogenic microbial community rather than a single pathogen, presenting many challenges for understanding the etiology and pathogenesis of this syndrome. A molecular version of Koch's postulates is presented for collecting evidence of causation for uncultivated microbes such as those linked to BV. There is new evidence suggesting that BV may be a biofilm condition in some women, which may contribute to poor treatment responses and high relapse rates. Understanding the bacterial biota of the human vagina is critical for optimizing reproductive health, and although many advances have been made, there is much that is unknown about how bacterial communities in the human vagina promote health and facilitate disease.

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Research Article

Temporal Shifts in Microbial Communities in Nonpregnant African-American Women with and without Bacterial Vaginosis

John Wertz,[1] Natasha Isaacs-Cosgrove,[2] Claudia Holzman,[3] and Terence L. Marsh[2]

[1] *Department of Biology, Calvin College, 3201 Burton Street, S.E. Grand Rapids, MI 49546, USA*
[2] *Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA*
[3] *Department of Epidemiology, Michigan State University, East Lansing, MI 48824, USA*

Correspondence should be addressed to Terence L. Marsh, marsht@msu.edu

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Bacterial vaginosis (BV) has been described as an increase in the number of anaerobic and facultatively anaerobic bacteria relative to lactobacilli in the vaginal tract. Several undesirable consequences of this community shift can include irritation, white discharge, an elevated pH, and increased susceptibility to sexually transmitted infections. While the etiology of the condition remains ill defined, BV has been associated with adverse reproductive and pregnancy outcomes. In order to describe the structure of vaginal communities over time we determined the phylogenetic composition of vaginal communities from seven women sampled at multiple points using 16S rRNA gene sequencing. We found that women with no evidence of BV had communities dominated by lactobacilli that appeared stable over our sampling periods while those with BV had greater diversity and decreased stability overtime. In addition, only *Lactobacillus iners* was found in BV positive communities.

1. INTRODUCTION

The relationship between biodiversity and ecosystem stability has been critically discussed and investigated over the past decade [1–10]. Most reports have provided evidence suggesting that greater biodiversity leads to greater system stability in the face of stress. A number of theoretical constructs have been created to account for the relationship between biodiversity and stability [3, 11, 12] in which high biodiversity within an ecosystem is frequently equated with a level of functional redundancy. Thus in periods of stress, the loss of a species is not catastrophic given a level of redundancy.

Microbial communities provide a remarkable system for investigating these relationships. Many apparently stable microbial communities are constructed of hundreds or thousands of species. Notable examples are the human intestinal microflora with an estimated 500–600 species [13, 14] and soil with an estimated 2000–3000 species/gram [15]. Perturbations resulting in significant community shifts have been detected in both of these communities (e.g., [16]), but their stability has not been carefully measured nor has the level of biodiversity been robustly correlated with stability. Interestingly, Fernández et al. [17] described a bioreactor with functional stability but apparent dynamicism in the phylogenetic composition of the community throughout the experiment. This is consistent with a level of functional redundancy among the species present that maintained the overall process in spite of phylogenetic shifts within the community.

From the perspective of the biodiversity-stability debate, the vaginal tract is an interesting ecosystem. In a large percentage of females the vaginal microbial community is relatively simple and dominated by one or several species of *Lactobacillus* [18–23]. However, when this simple community is replaced by bacterial vaginosis (BV), the shift is from the near monoculture of lactobacillus to a community with orders of magnitude greater phylogenetic diversity, especially in regards to Gram-positive anaerobes [19, 24–27]. Only a few investigators have addressed the stability of the community over time for either BV negative or BV positive females (e.g., [28, 29]).

In the work described herein, we present phylogenetic assessments of the vaginal microbial community from nonpregnant women. Multiple samples were taken from each woman on a monthly schedule, and the phylogenetic composition of the communities was determined by comparative sequence analysis of 16S rRNA gene libraries. Our goal was to compare the microbial community structure in BV positive

and BV negative women over time and examine whether diversity correlated with greater stability.

2. METHODS

2.1. Study sample

Vaginal samples used in this study were collected as part of a small, randomized clinical trial (RCT) of vaginal douching cessation. The primary goals of the RCT were to assess the acceptability of douching cessation and an at home data collection protocol over a four-month period. Secondary goals included describing BV presence/absence throughout the study period, and identifying factors associated with BV (e.g., phase of menstrual cycle, lifestyle). The study was conducted on a college campus and eligibility criteria included douching currently at least once per month and not being pregnant. Women were enrolled over a six-week period and total sample size was limited to the first 45 eligible women. At enrollment, women met with study personnel at the campus clinic to review and sign consent forms and to complete a baseline questionnaire. Participants were then randomized either to continue usual douching patterns or to refrain from all vaginal douching. At enrollment, women self-collected two swabs for baseline data on vaginal microflora. Thereafter, study participants were asked to complete a daily diary and self-collect three vaginal swabs a week (one on the weekend and two spread across the weekdays) for four months. Diaries included information about days of menses, sexual activity, contraceptive use, vaginal douching, vaginal symptoms, bathing, showering, illness, medications, and stress level. Diary sheets and slides were returned by mail weekly. Participants also returned to the campus clinic every two weeks at which time a swab for BV assessment was collected and vaginal pH was measured. Once per month an additional swab was collected, placed in sterile saline and frozen at -80°C.

For the current study, frozen vaginal fluid samples from seven women enrolled in the RCT were selected for further study. All seven women were African-American, and they had been assigned to the intervention arm (i.e., asked to refrain from vaginal douching) and reported that they did not use any form of hormone-based contraceptive. For comparison purposes, five of the seven women were selected because, throughout the four months of the RCT, all of their vaginal smears were negative for BV; the other two women were selected because they frequently showed evidence of BV during the same study period. Clone libraries of 16S rRNA genes were constructed from 2-3 monthly vaginal samples of each BV negative woman and 4 monthly samples from BV positive women.

2.2. Nugent scoring for BV

A single microbiologist with training in the Nugent method for scoring BV [30] evaluated all vaginal smears while blinded to the randomization assignment and data from the diaries. In a previous study with the same microbiologist and a second microbiologist, the kappa for BV positive (Nugent score ≥ 7) versus BV negative smears was .81 [31]. Nugent scores range from 0 to 10, the higher scores are indicative of more Gram-negative aerobes and Gram-positive anaerobes and fewer lactobacilli. A Nugent score of 0–3 is considered BV negative, 4–6 is intermediate, and 7–10 is BV positive.

2.3. Extraction of DNA and PCR amplification

Microbial DNA was extracted using MoBio Soil DNA extraction kits as follows. Frozen vaginal swabs were soaked in 70% ethanol overnight. The tip was removed from the tube and residual ethanol was squeezed out on the side of the tube. The swab tip was then cut off and placed into the MoBio extraction tube and stored at -20°C until extraction. The ethanol wash was centrifuged for 30 minutes at $10{,}000 \times$ g in a microfuge and the resulting pellet was resuspended in 200 μL of water and transferred to the MoBio extraction tube with the swab tip. The combined pellet and swab tip were lysed by bead beating for 1.5 minutes and then extracted according to the manufacturer's instructions. This protocol ensured that both free DNA derived from lysed cells and DNA from intact cells were collected from the samples. Pilot PCR reactions of 25 μL were performed using bacterial domain specific primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1389R (5′-AGC GGC GGT GTG TAC AAG-3′) [32]. The PCR reaction volume was 25 μL with 30 ng template DNA. Reactions contained 1X buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.25 mM of dNTPs, and 0.2 μM of each primer and 0.6 units of Taq polymerase (Invitrogen). Cycling was initiated with an initial denaturation of 3 minutes at 95°C followed by 25 cycles of 45 seconds at 95°C, 45 seconds at 56°C and 1 minute at 72°C, followed by a 5-minute extension at 72°C. PCR products were analyzed on agarose gels stained with ethidium bromide. Reactions with the appropriate size PCR product were cloned using Invitrogen's TOPO cloning kit. Putative clones with inserts were picked, screened and sequenced at the technology center at MSU. Sequences were deposited at GenBank (EF364727 to EF365525 (low Nugent scores) and EF365526 to EF366669 (high Nugent scores)).

2.4. Phylogenetic and statistical analyses

Each 16S rRNA gene clone was assigned a preliminary phylogenetic affiliation by sequence comparison to the Ribosomal Database Project II using the sequence match tool [33]. Sequences were checked for chimerae using the Chimera Check program [34], and sequences shorter than 550 nucleotides were removed. Sequences that were not clearly assigned at the genus level were compared to the Genbank nucleotide database using BLAST [35]. Sequences were aligned based on secondary structure to the 16S rRNA gene sequence database ssuJan03 in the ARB software package (http://www.arb-home.de/) using the Fast Aligner tool [36]. Unaligned or ambiguously aligned nucleotides were corrected manually. For all subsequent analyses, 503 unambiguously aligned nucleotides corresponding to positions 119 to 638 in *Escherichia coli* were used.

For phylogenetic analyses, when closely related sequences were not identified in the ARB database, relatives were found by a BLAST search of the Genbank database and incorporated into ARB. Phylogenetic trees were constructed using the neighbor-joining method with a Felsenstein correction. A minimum evolutionary distance method in PAUP* was used for bootstrap analysis of the same data.

Differences in the libraries were tested by pairwise comparison of PHYLIP-formatted distance matrices for each library using webLIBSHUFF version 0.96 [37], which combines preLIBSHUFF [38] and LIBSHUFF version 1.22 [39]. For further community analyses, the sequences were grouped into operational taxonomic units (OTUs) using DOTUR [39]. A distance of 3% was used to define an OTU, and is hereafter denoted as $OTU_{0.03}$. A 3% dissimilarity in 16S rRNA gene sequences is typically, though controversially, thought to represent a species-level delineation [40]. For each participant, the two-, three-, or four-clone libraries were combined and the Chao1 richness and Simpson diversity (D) estimators were calculated as implemented in the DOTUR program. The Simpson index of diversity was calculated as 1D. The Chao1 estimator, at an $OTU_{0.03}$ cutoff, can be thought to represent the estimated number of species in an environment. The Simpson index of diversity is an estimate that takes into account the richness as well as the evenness (number of each species). To obtain a quantitative measure of the $OTU_{0.03}$ similarity between libraries sampled from the same participant, the Yue and Clayton nonparametric maximum likelihood method was calculated using the SONS software [41].

3. RESULTS

In the seven women selected for this study, vaginal pH ranged from 4.0 to 5.8 and, as expected, pH was highest when BV was present (Table 1). For most women, samples were obtained at different menstrual weeks. Among BV positive women, there were no reports of antibiotic or antifungal use, and intercourse was infrequent in the week before vaginal sampling.

We evaluated the structure of the microbial communities from the seven women described in Table 1 with 16S rRNA gene libraries. A total of 20 libraries were made from both low- (5 women and 12 libraries) and high-(2 women and 8 libraries) Nugent scoring women. A total of 1,943 sequences were analyzed with library sizes raging from 50 to 170 clones (Table 1). *Lactobacillus* was the numerically dominant genus in 17 of the libraries. Three of the libraries from high-Nugent scoring women were dominated by *Leptotrichia/Sneathia, Prevotella,* and *Megasphaera,* respectively. In total, 28 genera were detected within the Firmicutes, Bacteriodetes, Actinobacteria, Proteobacteria, and Fusobacteria phyla. In the BV positive women, 20 different genera were detected while only 14 (of which 9 were singletons) were identified in the BV negative women.

Figure 1 presents the relative abundance of the detected genera in all of the libraries. The top 12-community composition profiles represent the communities from the five

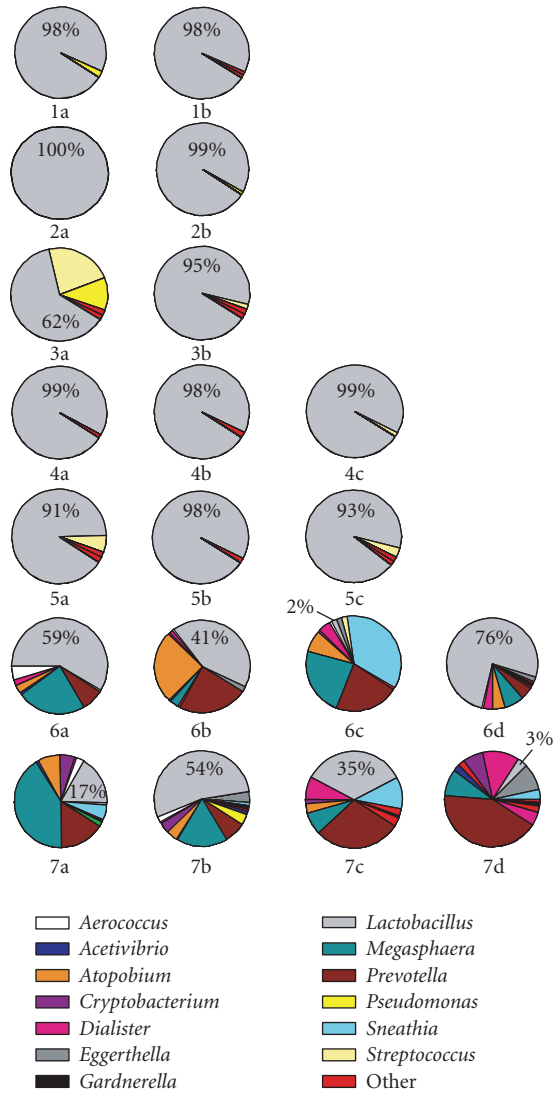

Figure 1: Pie chart representations of the vaginal microbial community structure between participants (top to bottom) and within each participant, over time (left to right) as inferred by 16S rRNA gene libraries. The percentage of each library consisting of clones related to members of the *Lactobacillus* genus is given.

individual women with low-Nugent scores. These communities were dominated by lactobacilli which usually constituted 91% of the community or greater. The exception to this was library 3a where *Lactobacillus* constituted only 62% of the clones.

The eight libraries derived from two women with high-Nugent scores are presented in the bottom two rows of community composition profiles in Figure 1. These revealed considerably more phylogenetic diversity than that found in low-Nugent scoring communities, consistent with the morphological basis of the Nugent scoring system and previously recorded observations [30]. Ten genera were identified in these libraries that were not detected in libraries from low-Nugent scoring women. Most of these genera displayed considerable volatility over time. For example, in

Table 1: Relevant clinical and 16S rRNA gene clone library information for the seven participants in this study.

Participant	Library	BV Score	pH	Menstrual cycle (week)	Frequency of intercourse (week prior)	Antibiotic/ antifungal (previous month)	No. of clones in library	Phylogenetic affiliation of dominant phylotype	Dominant phylotype (% of library)
1	1a	0	4.4	2	0	No	85	*Lactobacillus crispatus*	98
	1b	1	4.4	3	0	No	86	*L. crispatus*	98
2	2a	0	4.0	4	0	Antibiotic	50	*L. crispatus*	100
	2b	0	4.0	4	0	No	91	*L. crispatus*	99
3	3a	2	4.7	3	0	Antibiotic	53	*Lactobacillus gasseri*	62
	3b	4	4.7	3	0	No	58	*L. gasseri*	95
4	4a	1	4.4	4	4	Antifungal	79	*Lactobacillus iners*	99
	4b	2	4.7	4	0	Antifungal	50	*L. iners*	98
	4c	0	4.4	6#	1	Antifungal	74	*L. iners*	99
5	5a	0	4.0	4	3	No	53	*L. iners*	91
	5b	4	4.7	2	4	No	60	*L. iners*	98
	5c	0	*	5#	0	No	60	*L. crispatus*	93
6	6a	8	5.0	2	0	No	147	*L. iners*	59
	6b	8	5.8	4	1	No	170	*L. iners*	41
	6c	8	5.8	3	0	No	162	*Leptotrichia amnionii*	36
	6d	4	4.0	5#	0	No	159	*L. iners*	76
7	7a	8	5.0	3	0	No	119	*Megasphaera* sp.	62
	7b	9	5.0	3	0	No	165	*L. iners*	54
	7c	8	5.5	2	1	No	130	*L. iners*	35
	7d	8	5.5	1	0	No	92	*Prevotella buccalis*	40

*Missing data.
#Long menstrual cycle.

woman #6, the genus *Megasphaera* constituted 22%, 3%, 27%, and 10% of libraries A, B, C, and D, respectively. This irregular flux in clone numbers was also seen in *Prevotella* in woman number 7. Moreover, the lactobacilli were also volatile in clone numbers over time and were, in general, greatly reduced in numbers in women with high-Nugent scores. This is in contrast to libraries from low-Nugent scoring samples where lactobacilli were routinely high and constant in clone numbers over time.

To quantitate these diversity differences we applied a suite of ecological and statistical measurements to these libraries (Table 2). The Simpson's diversity index revealed at least a twofold difference between low and high-Nugent scoring communities while the Chao species richness similarly revealed substantial differences between these two groups. The Yue and Clayton analysis [41] measures library similarities. In this table, we calculate intra-woman library similarities and then compare these across the range of Nugent scores. All libraries with low- Nugent scores had high similarity (>79%) whereas the high-Nugent scoring libraries had low similarities (<44%). On visual inspection of these libraries, it was clear that there was structural instability in the community over time. Nugent scores did not reveal subtleties of phylogenetic composition as demonstrated by comparing community profiles 3a and 6d.

Analysis of the microbial communities among and between participants with high- and low-Nugent scores showed that approximately 95.0% (758 out of 799) of the clones from the low-Nugent scoring women were lactobacilli (Table 3). Of the remaining 5%, most were identified as streptococci (19 clones) or pseudomonads (9 clones). The genus *Lactobacillus* also contained the most number of clones of any other genus identified in the participants with high-Nugent scores, though the lactobacilli only accounted for 38.3% (438 out of 1144) of the total. A majority of the remaining clones grouped with the genera *Prevotella* (17.3%), *Megasphaera* (15.7%), *Atopobium* (7.5%), *Sneathia* (7.3%), *Dialister* (3.6%), and *Cryptobacterium* (2.4%). Of these, only *Sneathia* was not consistently present in all eight libraries (Figure 1). No clones belonging to any of these genera were obtained from participants with low-Nugent scores.

Table 2: Relationship between BV score and the diversity, richness, and stability of the vaginal microbial community.

Participant[1]	BV score[2]	Simpson diversity (1D)	Chao1 species richness	Library similarity (%)[3]
1 (2)	0.5	0.15	7	97.0 ± 1.9
2 (2)	0	0.03	4	100.0 ± 0.1
3 (2)	3	0.38	14	79.1 ± 8.6
4 (3)	1	0.03	7	100 ± 0.1
5 (3)	1.3	0.33	15	91.0 ± 5.0
6 (4)	7	0.75	27	43.1 ± 4.6
7 (4)	8.3	0.85	22	38.2 ± 6.9

[1]Numbers in parentheses represent the total number of clone libraries for that participant.
[2]Mean of BV scores given in Table 1.
[3]Calculated by the nonparametric maximum likelihood estimator of Yue and Clayton. Values ± SE.

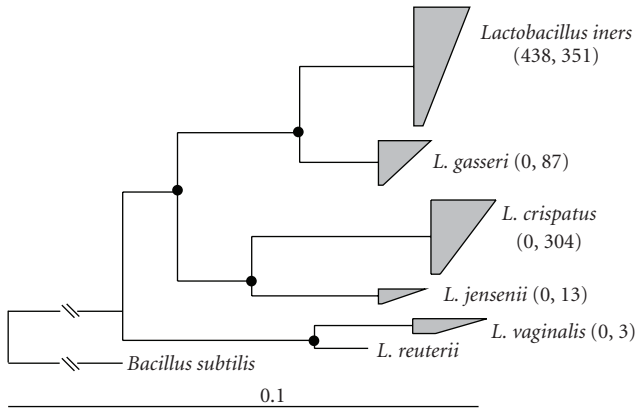

Figure 2: Neighbor joining based phylogeny of the 1,196 *Lactobacillus* 16S rRNA gene clones obtained in this study. Clones that were closely related to known *Lactobacillus* species were condensed into trapezia. Numbers in parentheses represent, for each group, the number of clones obtained from participants with bacterial vaginosis (left) and without bacterial vaginosis (right). The phylogeny is based on 503 unambiguously aligned nucleotides. Branch points with >75% conservation are represented with a closed circle; branch points with 50–74% conservation are shown with an open circle. Genbank accession numbers for reference species are shown in brackets. A 16S rRNA gene from *Bacillus subtilis* was used as the outgroup. Scale bar represents 0.1 change per nucleotide.

Among the two participants with high-Nugent scores, the distribution of *Prevotella*, *Atopobium,* and *Cryptobacterium* species was distinct (Figure 3). A majority of *Prevotella* clones from participant 6 grouped with *Prevotella bivia*, whereas those from participant 7 grouped most closely with *Prevotella buccalis*, *P. corporis* and *P. disiens*. Similarly, participant 6 had approximately three times the number of clones that grouped with *Atopobium vaginae* than did participant 7, whereas participant 7 had approximately three times the number of clones grouped with *Cryptobacterium curtum* than participant 6 (Figure 3). Both participant 6 and 7 had a similar overall distribution of species within the *Megasphaera* and *Dialister* genera.

As mentioned above, clones belonging to the genus *Lactobacillus* were the most abundant, irrespective of Nugent score. However, participants with low-Nugent scores had a diversity of *Lactobacillus* species that included *L. iners*, *L. gasseri*, *L. crispatus*, *L. jensenii*, and *L. vaginalis* (Figure 2) whereas libraries from participants with high-Nugent scores contained only *L. iners* (Figure 2).

4. DISCUSSION

Regarding the vaginal tract community structure of women with low-Nugent scores, our results were similar to previously reported studies [18–20, 23, 42]. All communities were dominated by Lactobacillus spp. Five different species were detected in the 758 *Lactobacillus* sequences including *L. iners, L. gasseri, L. crispatus, L. jensenii*, and *L. vaginalis*. Interestingly, we failed to detect any Lactobacillus sp. other than *L. iners* in BV positive women. Similar asymmetric distribution of lactobacillus species have been reported where *L. gasseri* and *L. iners* were "negatively correlated to each other" [43] or positively correlated with BV-associated bacteria [44]. Our results suggest that *L. iners* may be better adapted to the polymicrobial state of BV, including elevated pH.

Bacterial vaginosis has been described as a polymicrobial syndrome [19, 21, 25, 45, 46] with higher microbial diversity than what is perceived as the healthy ground state dominated by lactobacilli. Clinically it is characterized by a white discharge, an increase in pH and amine concentration, the appearance of clue cells, and a microbial community shift detected by Gram stain of smears from vaginal fluid [19, 21, 24–26, 45, 46]. Similar to previous work (e.g., [19, 47]) we detected greater species diversity in the BV positive subjects. In our 7 samples from the two BV positive women, we detected five clades within the *Prevotella* genus, the most abundant of the nonlactobacillus genera present in our libraries. Two of the *Prevotella* clades detected were present in both BV positive women while three were present in only one. This may reflect host differences that select for unique species or the consequences of sampling at nonsaturating levels. *Magasphaera* (2 clades, 180 clones), *Dialister* (2 clades, 41 clones), *Cryptobacterium* (1 clade, 27 clones) *Atopobium* (1 clade, 86 clones), *Eggerthella* (1 clade, 21 clones), and *Gardnerella* (1 clade, 7 clones) were also detected in BV positive women, although clone numbers were different. The

Table 3: Phylogenetic affiliation of 16S rRNA gene clones obtained from participants with and without bacterial vaginosis.

Phylum	Genus[1]	Participants with BV	Participants without BV	Total
Firmicutes				
	Lactobacillus	438	758	1196
	Megasphaera	180	0	180
	Dialister	41	0	41
	Streptococcus	3	19	22
	Acetivibrio	15	0	15
	Aerococcus	16	0	16
	Micromonas	8	0	8
	Gemella	5	1	6
	Veillonella	0	2	2
	Anaerococcus	1	1	2
	Peptoniphilus	0	1	1
	Helcococcus	1	0	1
	Staphylococcus	0	1	1
	Turicibacter	0	1	1
Bacteroidetes				
	Prevotella[2]	198	0	198
Actinobacteria				
	Atopobium	86	0	86
	Cryptobacterium	27	0	27
	Eggerthella	21	0	21
	Gardnerella	7	0	7
	Mobiluncus	5	0	5
Proteobacteria				
	Escherichia	0	1	30
	Serratia	2	0	23
	Pseudomonas	6	9	15
	Janthinobacterium	1	2	3
	Ralstonia	0	1	1
	Dechloromonas	0	1	1
	Klebsiella	0	1	1
Fusobacteria				
	Sneathia	83	0	83
Total		1144	799	1943
Total $OTU_{0.03}$		31	19	46

[1]Typically, clones with >94% 16S rRNA gene identity to the nearest cultivated relative were considered members of that genus.
[2]Clones grouped with the *Prevotella* genus had 92–94% 16S rRNA gene identity to their closest cultivated relative.
[3]Operational taxonomic units (OTUs) were calculated at a cutoff of 97% similarity.

surprising aspect of these studies was the volatility in clone demographics over time exhibited by the BV positive women. This suggests that in the case of women with clinically identified BV, the increase in diversity is accompanied by a decrease in community stability. It is possible that in spite of the phylogenetic volatility, the community function remains constant, as in the case of the previously cited bioreactors [17]. Other explanations are possible (see below). Nonetheless, in our BV positive women the phylogenetic composition changed dramatically over time in contrast to women with low-Nugent scores.

It is intriguing to consider the vaginal community in the light of ongoing discussions of biodiversity and stability of ecosystems, in part because of the demographic instability that we detect when the community is at its greatest diversity, in the BV positive women. While it seems (somewhat) intuitive to equate high biodiversity with a more resilient ecosystem; previous workers have concluded that there was "no such arbitrarily general rule" [48, 49]. Indeed, May points out that randomly constructed ecosystems "are more likely to lose species after disturbances than are simple ones" [49]. Moreover, in a separate paper May reported that

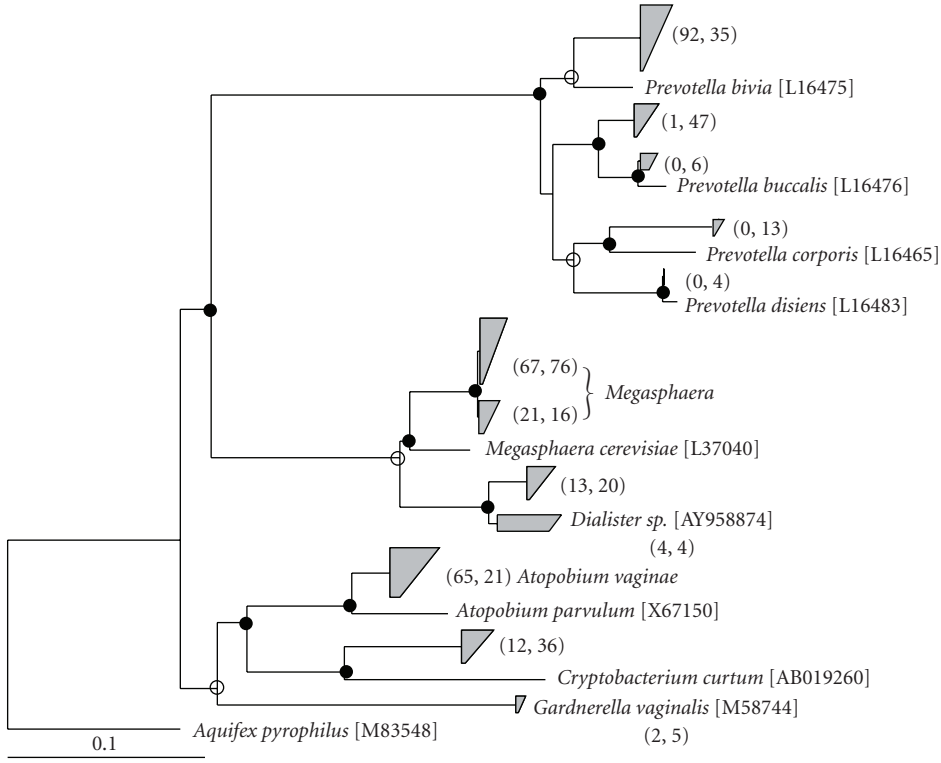

Figure 3: Neighbor joining-based phylogeny of 16S rRNA gene clones related to bacterial genera consistently present in participants with bacterial vaginosis. Closely related clones were condensed into trapezia with numbers in parentheses representing, for each group, the number of clones obtained from participant 6 (left) and participant 7 (right). The phylogeny is based on 503 unambiguously aligned nucleotides. Branch points with >75% conservation are represented by a closed circle. Genbank accession numbers for reference species are shown in brackets. A 16S rRNA gene from *Aquifex pyrophilus* was used as the outgroup. Scale bar represents 0.1 change per nucleotide.

simple nonlinear difference equations that describe growth can produce stable cycles as well as apparent chaotic regimes [50]. Hence, the community instability in the BV positive state that we observed could be more a reflection of a randomly assembled community and/or the composite of populations with nonoverlapping growth curves.

BV can be a recalcitrant condition even in the face of clinical treatment [25, 26, 46]. While the molecular approaches of microbial ecology have provided considerable insight into the phylotypes present [18–20, 23, 28, 42, 44], we remain somewhat distant from a complete ecological description of the vaginal community that includes the host genotypic variability, environmental influences, a complete description of the community including eukaryotes, bacteria and viruses [51] and critical interactions between species, not to mention prevailing nutrient sources and food webs [52]. It is encouraging that some investigators have identified strong correlations between certain bacterial phylotypes and BV (e.g., [19]). In addition, the hormonal milieu appears to influence vaginal microflora, as evidenced by a lower prevalence of BV in women exposed to exogenous hormones [31, 53] and a higher prevalence of BV in the first week of the menstrual cycle [31, 54]. In this study of seven selected participants, we specifically chose women who were unexposed to exogenous hormones and had consistent BV scores (i.e., primarily negative/intermediate or primarily positive) irrespective of the timing in the menstrual cycle. Moreover, we have come to view the syndrome as an ecosystem gone awry and currently efforts are being directed at identifying the conditions or events that cause community shifts [25, 26, 46]. While this ecosystem approach is more complex, it may prove more productive than pathogen hunting.

Our report is a preliminary study of relatively few women sampled over time where community structure was determined using culture independent techniques. We recognize the potential biases that can arise from PCR amplification and library construction [55] including primer bias. The latter is of particular concern because some phylogenetic groups can be missed entirely by poorly matched primer sets. For example Frank et al. [56] and Verhelst et al. [42] recently demonstrated that detection of *Gardnerella*, a genus frequently associated with BV (e.g., [42]), can be strongly influenced by primer selection. While our primer set did pick up *Gardnerella* sequences, the abundance may have been influenced by primer bias. However, in spite of these limitations we have identified substantial diversity within the *Prevotella* clones, an asymmetric distribution of the lactobacilli species and large demographic shifts over time in BV positive women.

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Review Article

Vaginal Microbiota and the Use of Probiotics

Sarah Cribby,[1,2] Michelle Taylor,[1,2] and Gregor Reid[1,2]

[1] *Canadian Research and Development Centre for Probiotics, Lawson Health Research Institute, 268 Grosvenor Street, London, ON, Canada N6A4V2*
[2] *Department of Microbiology and Immunology, University of Western Ontario, London, ON, Canada N6A4V2*

Correspondence should be addressed to Gregor Reid, gregor@uwo.ca

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The human vagina is inhabited by a range of microbes from a pool of over 50 species. Lactobacilli are the most common, particularly in healthy women. The microbiota can change composition rapidly, for reasons that are not fully clear. This can lead to infection or to a state in which organisms with pathogenic potential coexist with other commensals. The most common urogenital infection in premenopausal women is bacterial vaginosis (BV), a condition characterized by a depletion of lactobacilli population and the presence of Gram-negative anaerobes, or in some cases Gram-positive cocci, and aerobic pathogens. Treatment of BV traditionally involves the antibiotics metronidazole or clindamycin, however, the recurrence rate remains high, and this treatment is not designed to restore the lactobacilli. In vitro studies have shown that *Lactobacillus* strains can disrupt BV and yeast biofilms and inhibit the growth of urogenital pathogens. The use of probiotics to populate the vagina and prevent or treat infection has been considered for some time, but only quite recently have data emerged to show efficacy, including supplementation of antimicrobial treatment to improve cure rates and prevent recurrences.

1. THE MICROBIOTA OF THE VAGINA

The microbial species that inhabit the vaginal tract play an important role in the maintenance of health, and prevention of infection. Over 50 microbial species have been recovered from the vaginal tract [1–3]. These species do not exist independently, and studies in vitro and in humans have shown that a multispecies microbiota, usually associated with bacterial vaginosis (BV), are present in dense biofilms [4–7], while a lactobacilli dominant microbiota can be sparsely distributed on the epithelium [4, 5, 8]. In comparison, the gut is populated with more than 800 species of microbes, the majority of which are excreted in feces, and a number of which are well equipped to be pathogenic. Despite the close proximity of the vagina to the anus, the diversity of microbes present in the vagina is much lower than in the gut. The reason for this lower diversity is still unclear, but may involve poor receptivity of the vagina, different nutrient availability compared to the gut, and competition with indigenous organisms. Some species found in the gut, such as *E. coli* and *Streptococcus,* can also be found in the vagina, indicating the proper receptors, nutrients, and oxygen tension are present for these organisms to grow.

Different methodologies are being used to identify the composition of the vaginal microbiota. Each has its strengths and weaknesses. Culture-based methods allow strains to be identified and used for further experimentation. However, as there remains a major defect in our ability to grow many bacterial species, we must rely on nonculture methods to identify the breadth of vaginal microbiota. This has been achieved by analyzing their ribosomal DNA sequences [3, 9], using a combination of PCR and denaturing gel gradient electrophoresis (DGGE) [2, 5, 10–12], and by using degenerate, universal polymerase chain reaction primers to amplify an approximately 555 base-pair regions of the universal chaperonin-60 gene [13].

The species that are present in the vaginal mucosa vary between premenopausal woman and those who have gone through menopause. The microbiota of healthy premenopausal woman is generally dominated by *Lactobacillus* species, the most common of which are *L. iners*, *L. crispatus*, *L. gasseri*, *L. jenesenii,* followed by *L. acidophilus, L. fermentum, L. plantarum, L. brevis, L. casei, L. vaginalis, L. delbrueckii, L. salivarius, L. reuteri,* and *L. rhamnosus* [2, 5, 9–16]. As more studies are performed on the vaginal organisms in healthy women, it is possible that some women

will be identified, who do not have a lactobacilli-dominated microbiota [17]. However, until we know more about the dynamics of such a population, and are sure that it does not increase the risk of the disease, lactobacilli will remain the organisms of most importance to vaginal health.

Factors such as hormonal changes (particularly estrogen), vaginal pH, and glycogen content can all affect the ability of lactobacilli to adhere to epithelial cells and colonize the vagina [16]. The menstrual cycle can also cause changes in the vaginal microbiota, with high concentrations of estrogen increasing adherence of lactobacilli to vaginal epithelial cells [18]. With the decrease in estrogen levels associated with menopause, there is also a decrease in lactobacilli present in the vaginal tract of postmenopausal women [5, 11, 12, 19]. Postmenopausal women are also more susceptible to urogenital infections, supporting the theory that colonization of the vagina by commensal lactobacilli serves as a protection from these pathogens [19, 20]. Although the methods by which these organisms do this are still unclear, it appears to involve an ability to adhere to and to populate the vaginal epithelium and mucin layer, to inhibit pathogens from taking over [21–24], to reduce pathogen virulence [25, 26], and to modulate host defenses [27].

Hormone replacement therapy (HRT) alters the bacterial profile of the vaginal tract of postmenopausal women, and restores a lactobacilli-dominated state, as well as reduces the incidence of urinary tract infections (UTI) [19]. In a study of women taking combination conjugated equine estrogen and progesterone HRT, only 1 to 3 species of bacteria, mainly *Lactobacillus*, were detected in the vaginal mucosa of 87% of the women [5]. In postmenopausal women not receiving HRT, almost all subjects had vaginal mucosa populated with more than 1 organism, many of which had pathogenic potential such as *Bacteroides, Prevotella,* and *Gardnerella*, associated with bacterial vaginosis (BV), and *E. coli* and *Enterococcus,* associated with UTI [5].

While a vaginal tract dominated by lactobacilli appears to protect the host against some vaginal infections, it does not fully prevent colonization by other species. Pathogens are still able to coexist with these commensal organisms, as shown by Burton and Reid [10], where *G. vaginalis*, a pathogen associated with BV, was detected in a vaginal sample which also contained a species of *Lactobacillus*. Interestingly, *G. vaginalis* was displaced beyond detectable limits for 21 days, following a single intravaginal instillation of probiotic lactobacilli [11]. As more and more studies are uncovering the diversity microbiota of the vagina, it seems apparent that the balance between a healthy and diseased state involves some sort of equilibrium or see-saw effect, which can swing in either direction depending on a number of factors, such as hormone levels, douching, sexual practices, as well bacterial interactions and host defenses [20, 21].

Witkin et al. [28] have proposed that innate immunity plays an important role in the switch to BV from a healthy state. The mechanism they propose is through microbial-induced inhibition of Toll-like receptor expression and/or activity blocking proinflammatory immunity, as well as a lack of 70-kDa heat-shock protein production, and a deficit in vaginal mannose-binding lectin concentrations decreasing the capacity for microbial killing. Three recent studies have provided further insight into the host's role. In a study of women susceptible to UTI, it was discovered that immunological defects in peripheral blood coexisted with a persistently aberrant microbiota (Kirjavainen et al. [29]). In postmenopausal women, BV was associated with apparent reduced expression of host antimicrobial factors [30]. When probiotic *L. rhamnosus* GR-1 was administered to the vagina of premenopausal women, it resulted in 3 536 gene expression changes and increased expression levels of some antimicrobial defenses [31].

2. NONSEXUALLY TRANSMITTED INFECTIONS OF THE VAGINAL TRACT AND INTERFERENCE BY LACTOBACILLI

Pathogenic organisms are able to infect the vagina, with BV, yeast vaginitis, and UTIs causing an estimated one billion or more cases per year [32–35]. While there is some evidence that the causative organisms can be transmitted by sexual partners, these conditions will be discussed here as nonsexually transmitted. Other reviews adequately cover sexually transmitted infections [36, 37].

Yeast vaginitis is characterized by white discharge, local itching, and irritation. The majority of cases are caused by *Candida albicans*, but *C. glabrata*, *C. krusei*, and *C. tropicalis* can be problematic [35]. It is diagnosed by microscopic detection of dense numbers of yeast cells on a vaginal smear, and by physical examination and the presence of a white, mucous-like yeast discharge. Of note, lactobacilli are often found in patients with yeast vaginitis, therefore, the induction of infection does not appear to require the yeast displacing or killing off the lactobacilli.

Urinary tract infections occur when pathogenic bacteria ascend from the vagina and replicate on, and sometimes within, the bladder urothelium [32, 38, 39]. These infections are frequent among women, with an estimated 50% suffering at some time in their life. Symptoms and signs include suprapubic pain, dysuria, pyuria, frequency and painful micturition, and occasionally hematuria. Asymptomatic bacteriuria is also a common occurrence, particularly amongst the elderly. The most frequent pathogen is *E. coli*, followed by *Enterococcus faecalis,* and *Staphylococcus saprophyticus* [39]. Diagnosis can be achieved by presence of symptoms and signs, and urine samples containing over 10^3 organisms/mL of the pathogens. In a portion of patients, the *E. coli* invade the bladder epithelium and form dense biofilms that are recalcitrant to antibiotics [40]. In women with no history of UTI, their vagina and perineum is most commonly colonized by lactobacilli [20], while in women with recurrent UTI there is an inverse association between lactobacilli and *E. coli* [41], suggesting that lactobacilli play a role in preventing infection.

The most common urogenital disorder in women of reproductive age is BV, a condition discussed above. The vaginal microbiota of BV patients typically contains a broader range of species than found under healthy conditions, with *Atopobium vaginae*, *Bacteroides* spp., *Gardnerella vaginalis*, *Mobiluncus*, *Megasphera*, *Mycoplasma hominis*, *Peptostreptococcus*, and *Prevotella* being particularly

prevalent [3, 42–46]. BV is associated with multiple species of bacteria that occur in 90% of the cases, and essentially consists of an elevated vaginal pH (>4.5) and depletion of lactobacilli. It affects women of all age groups, and is often asymptomatic [47]. When symptoms and signs do occur, they include fishy odor, discharge, and vaginal pH above 4.5 [48]. Indeed, this formed the basis of the often-used Amsel criteria for BV diagnosis: presence of at least 3 of the following criteria: (1) release of an amine or fishy odor upon addition of 10% potassium hydroxide, (2) a vaginal pH higher than 4.5, (3) detection of at least 20% of clue cells (which are vaginal cells colonized by Gram-negative rods), and (4) a milky homogeneous vaginal discharge [48]. A Gram-staining method called the Nugent score has also been used [8]. It comprises a scoring system based on the morphology of bacteria present in vaginal swab samples. A normal score is given to samples showing predominantly Gram-positive rods indicative of lactobacilli, while the presence of predominantly small and curved shaped Gram-negative rods and Gram-positive cocci, along with the absence of lactobacilli, is indicative of BV. The BVBlue test is another kit used to diagnose BV, and works by detecting sialidase produced by pathogens associated with the condition [49, 50]. Of note, aerobic vaginitis has also been described in which the vagina is colonized by organisms such as *E. coli* and enterococci [51]. During pregnancy, BV can increase the risk of preterm labor and low birth weight [52, 53]. Other problems associated with BV include pelvic inflammatory disease, UTI, and increased susceptibility to sexually transmitted diseases, including HIV [54–57].

The organisms associated with BV form dense biofilms on the vaginal epithelium, and these are associated with increased resistance to lactobacilli-produced lactic acid and hydrogen peroxide (H_2O_2) which are normally antagonistic to planktonic organisms [58]. The biofilms are also able to induce host expression of certain inflammatory factors, such as IL-1 and IL-8 [59]. It is not currently known whether the production of H_2O_2 by lactobacilli has a clinically protective role against BV. The increased prevalence of H_2O_2-peroxide producing vaginal lactobacilli in healthy women has been given as a reason to believe that it is a protective factor [60], however, those studies used culture to recover the lactobacilli, and arguably had they used nonculture methods, *L. iners* would have been the most commonly isolated and it does not appear to produce H_2O_2. It is possible to isolate *L. iners* by culture, but it requires selective media and extensive incubation. The same group found that women with the H_2O_2-producing vaginal *L. crispatus* or *L. jensenii* had a significantly lower incidence of BV than women with a different vaginal flora [14]. However, Alvarez-Olmos et al. [61] and Rosenstein et al. [62] found H_2O_2-producing lactobacilli in 85% and 91.7%, respectively, of women with BV. It could be argued that the high prevalence of H_2O_2-producing lactobacilli shows that this compound is not protective [32]. Either way, it is difficult to make a definitive conclusion.

McLean and McGroarty [63] conducted an in vitro study showing that increasing culture pH reduced the bacteriostatic effects of *L. acidophilus* on *G. vaginalis* NCTC 11292 by 60%; a 30% reduction in bacteriostatic effects was seen when catalase was introduced to degrade H_2O_2. Klebanoff et al. [64] found that the toxicity of H_2O_2-producing lactobacilli was inhibited by the presence of catalase but lactobacilli that do not produce H_2O_2 were not affected. High concentration of H_2O_2-producing lactobacilli inhibits the growth of both *G. vaginalis* and *Bacteroides bivius*. However, low concentrations of H_2O_2-producing lactobacilli must be combined with myeloperoxidase and chloride in vaginal mucus, to be toxic toward *G. vaginalis*, with a maximum toxicity in a pH range of 5 to 6. A pH of $\leq$4.5 inhibited the growth of *G. vaginalis* on its own and this effect increased with the addition of the above combination. Suffice to say, H_2O_2 is likely one of several factors involved in competition with other organisms in the vagina.

3. PROBIOTICS TO PREVENT AND TREAT UROGENITAL INFECTIONS

As antimicrobial treatment of urogenital infections is not always effective, and problems remain due to bacterial and yeast resistance, recurrent infections [65, 66], as well as side effects, it is no surprise that alternative remedies are of interest to patients and their caregivers. It is assumed that recurrences are due to antimicrobials failing to eradicate the pathogens, perhaps because of biofilm resistance, or that the virulent organisms come back from their source (the person's gut, or a sex partner) and attack a host whose defenses are suboptimal. Young girls who suffer from UTI are more likely to have repeated episodes in adulthood, and overall many UTI, BV, and yeast vaginitis patients will have a recurrence [21, 67]. Recurrent infection may also be due to the elimination of the commensal organisms in the vagina by the antimicrobial, thereby increasing susceptibility to recolonization by pathogens [68, 69]. This is one of the main reasons for considering the use of probiotics, to replenish the commensal microbes as a way to lower the risk of reinfection. In a study of 120 children with persistent primary vesicoureteral reflux, *L. acidophilus* treatment daily was as effective as trimethoprim/sulfamethoxazole in reducing the rate of UTI ($P = .926$), suggesting that probiotics could provide a prophylactic option [70].

The route of delivery of probiotic lactobacilli has intuitively been via direct instillation into the vagina. For example, the weekly application of *L. rhamnosus* GR-1 and *L. fermentum* B-54 was shown to reduce UTI recurrences from an average of 6 to 1.6 per year [71]. The ability of a given strain of lactobacilli to adhere to vaginal cells was considered an advantage in temporarily populating the vaginal [71, 72] and creating an environment conducive to the restoration of the host's indigenous lactobacilli rather than a return of pathogens. The adhesion of lactobacilli to the uroepithelium varies among species and strains, as shown by in vitro studies [72], and may be mediated by glycoprotein and carbohydrate adhesins binding to glycolipid receptors [73]. Still, it is unclear the extent to which a difference in in vitro adhesion, say of 10 per cell, means that an organism will succeed or fail to protect the host if instilled into the vagina. Thus, adhesion per se is not the definitive criteria to predict success. Once

administered in a viable count of one billion or more, *L. rhamnosus* GR-1 and *L. reuteri* (formerly *fermentum)* RC-14 have been found to be detectable for three weeks or more, depending on the host [74, 75]. This implies a correlation between in vitro adherence and in vivo presence.

The concept of delivering lactobacilli orally to repopulate the vagina was first reported in 2001 [76], and based upon the question "if urogenital pathogens can do this, why cannot lactobacilli"? The organisms were delivered in a milk base and shown to be recovered from the rectum [77]; therefore supporting the concept that ingested strains could pass through the intestine, reach the rectum, and potentially ascend to the vagina. This was confirmed independently by others [78].

In order to conduct clinical studies with the view of providing more women with access to these strains, a two-year shelf life capsule formulation was then developed and used successfully in a number of studies. An oral dose of over one billion organisms per day was found to maintain a lactobacilli-dominated vaginal presence [79]. The time for this intervention to affect the vaginal tract is obviously longer than direct vaginal instillation, and will depend on viability of the strains as they pass through the stomach and gut [78]. In addition, the load of lactobacilli that can be delivered this way is clearly lower than via vaginal administration. However, an advantage of the oral approach may be the ability of the lactobacilli to reduce the transfer of yeast and pathogenic bacteria from the rectum to the vagina [80], which could potentially lower the risk of infection. In that randomized, placebo-controlled trial of 64 healthy women, 37% of the patients in the *L. rhamnosus* GR-1 and *L. reuteri* RC-14 probiotic group had a lactobacilli-dominated normal vaginal microbiota restored from a BV vaginal flora compared to 13% in the placebo group ($P = .02$). At both the 28-day and 60-day test points, women in the lactobacilli treatment group had a greater number of vaginal lactobacilli than women in the control group ($P = .08$ and $P = .05$, resp.) as shown by microscopy and culture. The ability of this oral probiotic therapy to create a lactobacilli normal flora and convert some subjects from a BV status to normal [79] goes beyond the proof-of-concept stage and provides a method for women to help maintain vaginal health. Failure of *L. rhamnosus* GG to be effective, at least in one small study [79], emphasizes the strain-specific aspects of probiotic use. Thus, one cannot and should not utilize the data from one strain to infer that another untested strain will provide the same benefits.

The mechanisms whereby lactobacilli function as anti-infective defenses are still not fully understood. As discussed above, this may involve production of antimicrobial factors [81], and maintenance of a vaginal pH of ≤4.5. It could also be due to biosurfactants which alter the surrounding surface tension and reduce the ability of a wide range of pathogens to adhere [82, 83]. This might explain the relatively sparse coverage of epithelial cells noted in healthy women [8]. In addition, lactobacilli have been shown to bind (coaggregate) some pathogens and this may be a means to block their adhesion, kill them through production of antimicrobials, and prevent their spread to other areas of the vagina and bladder [84]. Among 10 strains of lactobacilli being evaluated for use in a probiotics tablet, Mastromarino et al. [85] found, in vitro, that *Lactobacillus gasseri* 335 and *Lactobacillus salivarius* FV2 were able to coaggregate with *G. vaginalis*. When these strains of lactobacilli were combined with *Lactobacillus brevis* CD2 in a vaginal tablet, adhesion of *G. vaginalis* was reduced by 57.7%, and 60.8% of adherent cells were displaced. Boris et al.found that the adherent properties *G. vaginalis* were similarly affected by *Lactobacillus acidophilus* [73].

It has been known for some time that *Lactobacilllus* produce bacteriocins that can inhibit the growth of pathogens, including some associated with BV, such as *G. vaginalis* [86]. Only relatively recently has a study shown in animals that bacteriocin production might have an effect in vivo. A stable mutant of *Lactobacillus salivarius* UCC118 that did not produce a specific bacteriocin was unable to protect mice against *Listeria* intestinal infection, while the wild type did, thereby leading the authors to conclude that bacteriocin production can be a primary mediator of anti-infective defense [87].

Relatively few studies have attempted to prevent urogenital infection using probiotics. Shalev et al. [88] assessed 46 premenopausal women with ≥4 episodes of BV and/or vaginal candidiasis in the previous year, to compare the recurrence of BV using a probiotic yoghurt versus one that was pasteurized. Patients were not receiving long-term antibiotics or immunosuppressive therapy and had not consumed yoghurt prior to the commencement of the study. They were randomly assigned to one of two treatment groups and ingested 150 mL of either pasteurized yoghurt ($n = 23$) or yoghurt containing *L. acidophilus* at $> 1.0 \times 10^8$ colony-forming units ($n = 23$). Yoghurt was consumed daily for two months followed by two months of no yoghurt. There was a 60% reduction in BV episodes among patients consuming probiotic yoghurt after one month while only a 25% reduction occurred in subjects who received pasteurized yoghurt ($P = .004$). After two months of yoghurt consumption, the results were similar; however, 25% of patients from both groups had left the study. Product integrity was only assessed prior to the study and no adverse effects were reported.

Neri et al. [89] studied 84 women in the first trimester of pregnancy to observe the effects of probiotic-containing yoghurt on BV. The subjects were randomized to one of three treatment groups: inserting a tampon containing 5% acetic acid ($n = 32$), a 10 to 15 mL vaginal douche containing $> 1.0 \times 10^8$ colony-forming units/mL of *L. acidophilus* ($n = 32$), or no treatment ($n = 20$). Both active treatments were administered twice a day for one week. Amsel criteria (three of five findings: release of an amine fishy odor; release of amine odor after the addition of 10% potassium hydroxide; vaginal pH greater than 4.5; clue cells in the vaginal fluid; milky homogenous vaginal discharge) were absent in 88%, 38%, and 15% of subjects who received intravaginal lactobacilli, acetic acid tampons, and placebo, respectively, after 30 days. There was a significant difference in the cure rate between probiotic and control groups ($P < .005$), and lactobacilli and acetic acid groups ($P = .004$).

Fredricsson et al. [90] conducted an open-label trial to compare the cure rates of 61 women with BV given one of four intravaginal products. Patients were diagnosed with BV if ≥3 Amsel criteria were present. Each of the four treatments that patients were randomized to receive was administered twice a day for seven days: 5 mL of fermented milk containing between $5.0 \times 10^8$and 2.0×10^9 colony-forming units/mL of *L. acidophilus* NCDO 1748 ($n = 13$), 5 mL of acetic jelly ($n = 15$), 5 mL of estrogen cream ($n = 16$), or 500 mg metronidazole vaginal tablets ($n = 15$). BV was considered to have been cured if ≤1 Amsel criterion was present at 4 and 8 weeks. After both 4 and 8 weeks from the initiation of treatment, the cure rates in the metronidazole, acetic acid, probiotic, and estrogen groups were 93%, 18%, 7%, and 6%, respectively; no statistical analysis was reported. In this case, the so-called probiotic was not effective. No information about the strain was provided.

The cure rates of BV in 57 women with a mean age of 24 were studied following treatment with either "probiotics" or placebo in a double-blind trial [91]. Subjects were randomized to receive either a vaginal suppository containing $1.0 \times 10^{8\text{-}9}$ colony-forming units of *L. acidophilus* ($n = 28$) or placebo ($n = 29$). The vaginal suppositories were administered twice a day for 6 days. Symptom resolution, which was not clearly defined, was used to evaluate the cure of BV. At 7–10 days after the commencement of treatment, BV symptoms were absent in 57% of women in the probiotic group and 0% of women in the placebo group ($P < .005$). After 20 to 40 from the initiation of treatment, the cure rate in the probiotic group fell to 21% and remained at 0% in the placebo group (p = NS). This poorly conceived study is hard to interpret and is insufficient to verify efficacy of the product.

Eriksson et al. [92] studied how lactobacilli augmented antibiotics in curing BV through a double-blind, placebo-controlled trial including 187 women with a median age of 32 over two menstrual periods. Open-label treatment with 100 mg/d of clindamycin was administered to all patients for 3 days. The subjects were then randomized to one of two treatment groups which required at least five tampons to be inserted during the next menstrual period. The treatment groups were placebo tampons ($n = 96$) and tampons impregnated with *L. fermentum, L. gasseri*, and *L. rhamnosus* at 1.0×10^8 colony-forming units per tampon ($n = 91$). Cure rates of BV were assessed by the absence of Amsel criteria after the second menstrual period in both the probiotic and placebo groups, and found to be 56% and 62%, respectively, (p = NS). Infection with *Candida* was reported in 14.3% of subjects in the probiotic group and 13.5% of patients in the placebo group. The viable number of bacteria per tampon diminished to 10^6 colony-forming units by the end of the study. In short, this product was not successful. The rationale for administering lactobacilli during menses could be questioned, as it exposes the users' blood stream directly to the organisms, and the flushing effect of menstruation may be nonconducive to lactobacilli repopulating the vagina.

A comparison of intravaginal probiotics and metronidazole gel in treating 40 women (ages 18 to 50) with BV was conducted by a single-blind study by Anukam et al. [93]. The presence of ≥3 Amsel criteria, a Nugent score of ≥7, and a positive sialidase test led to a diagnosis of BV. Patients were randomized to one of two treatment groups for five days. They either inserted an intravaginal capsule with *L. rhamnosus* GR-1 and *L. reuteri* RC-14 at 1.0×10^9 colony-forming units nightly ($n = 20$) or applied a 0.75% metronidazole gel twice daily ($n = 20$). A Nugent score of ≤3 at 30 days indicated a cure of BV. A BV cure rate of 88% in the probiotic group and 50% in the metronidazole group was found (p = NS). Treatment was prematurely discontinued by patients in both the metronidazole and probiotic groups at 10% and 15%, respectively. This study, albeit small in size, showed the potential of probiotics to cure BV.

The efficacy of combining probiotics or placebo with oral metronidazole was assessed in 125 women aged 18 to 44 [94]. Oral metronidazole was administered at 500 mg twice daily to all patients for 7 days, and they were randomized to receive twice-daily oral capsules containing either a placebo ($n = 60$) or *L. rhamnosus* GR-1 and *L. reuteri* RC-14 at 1.0×10^9 colony-forming units ($n = 65$) for a total treatment duration of 30 days. At the end of 30 days, BV was considered absent if the patient had a negative sialidase test and a Nugent score of <3. This was the case in 40% of placebo and 88% of probiotic subjects ($P < .001$). If an intermediate Nugent score was regarded as "cure of BV", the cure rate was 100% with metronidazole and probiotics versus 70% with metronidazole and placebo. This study is important as it implies that probiotics can augment the effects of antibiotics in treatment of disease. Further studies have confirmed this effect, but are awaiting publication.

4. POSSIBLE NEGATIVE EFFECTS OF PROBIOTIC USE

Annually, over one billion doses of probiotics are administered worldwide, and those administered for urogenital health have been well tolerated [11, 75, 93–96]. In addition, the mouth, gastrointestinal tract, and female genitourinary tract are inhabited by *Lactobacillus* [96]. Yet, endocarditis and bacteremia caused by lactobacilli are extremely rare. Most cases occur in patients with chronic diseases or debilitating conditions that provide direct access to the bloodstream from a leaky gut. Only 1.7% of 241 cases of bacteremia, endocarditis, and localized infections associated with *Lactobacillus* that were investigated by Cannon et al. were considered to have a possible link with heavy consumption of dairy products [97]. Only one case had a *Lactobacillus* isolate that was indistinguishable from a probiotic strain. There was no connection between the species of *Lactobacillus* isolated and the type of infection or mortality. A recent study that directly instilled a six-strain bacterial product into the intestine of patients with severe, potentially fatal pancreatitis portrayed probiotics as being dangerous [97]. However, the product had never been proven to be probiotic, it was administered as a drug unlike 99.9% of probiotics, the randomization process led to patients with multiorgan failure being given large doses of live bacteria, and the authors failed to provide a rationale for the study in an appropriate animal model. All this led to unwarranted adverse publicity for the field of probiotics [98].

Nevertheless, safety of probiotic use must continually be monitored and considered when doing clinical studies. The potential for transfer of antibiotic resistance is one factor to consider, although it remains to be proven that probiotics have contributed in any way to drug resistance, or disease. Rather, the overuse of antibiotics, especially in livestock feed and long-term prevention of infection, remains a root cause of the increasing concerns over drug resistance. Efforts to substitute prophylactic antibiotics with probiotics, especially in children with recurrent UTI [70] and perhaps some patients preparing to undergo surgery [99], are worthy of pursuit.

5. CONCLUSION

Molecular methodologies are providing a greater understanding of the dynamic microbial presence, both short and long term, in the vagina. The defenses of the host which include some of these microbes perform a remarkable function given the opportunity of pathogens to cause infection. The use of probiotic lactobacilli to prevent infection has a good rationale, and an excellent safety record, but so far only a few strains have been clinically proven to be effective, in particular to prevent BV. It is critically important that strains be characterized and tested clinically using the delivery system of choice (oral, vaginal, dried powder, or in suspension). An advantage for women is that they can self-administer the probiotics. Many more studies are needed to optimize the defensive properties of the vaginal microbiota, but the potential remains that the health of many women can be improved by probiotic intervention.

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Review Article

Probiotics and Gastrointestinal Infections

Robert A. Britton[1] and James Versalovic[2]

[1] *Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI 48824, USA*
[2] *Departments of Pathology, Baylor College of Medicine and Texas Children's Hospital, 6621 Fannin Street, MC 1-2261, Houston, TX 77030, USA*

Correspondence should be addressed to Robert A. Britton, rbritton@msu.edu

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Gastrointestinal infections are a major cause of morbidity and mortality worldwide, particularly in developing countries. The use of probiotics to prevent and treat a variety of diarrheal diseases has gained favor in recent years. Examples where probiotics have positively impacted gastroenteritis will be highlighted. However, the overall efficacy of these treatments and the mechanisms by which probiotics ameliorate gastrointestinal infections are mostly unknown. We will discuss possible mechanisms by which probiotics could have a beneficial impact by enhancing the prevention or treatment of diarrheal diseases.

1. INTRODUCTION

Within the microbiota, individual bacteria containing important genes may benefit the host in different ways. As one considers the vast community of commensal microbes, subsets of these organisms may have important physiologic benefits for the host in the context of human nutrition and host:microbe interactions. Probiotics may stimulate immunity, regulate immune signaling pathways, produce antipathogenic factors, or induce the host to produce antipathogenic factors. Probiotics may produce secreted factors that stimulate or suppress cytokines and cell-mediated immunity. These factors may also interfere with key immune signaling pathways such as the NF-κB and MAP kinase cascades. Probiotics may produce factors that inhibit pathogens and other commensal bacteria, effectively enabling these microbes to compete effectively for nutrients in complex communities. Microbes that produce antipathogenic factors may represent sources of novel classes of antimicrobial compounds, and these factors may be regulated by master regulatory genes in particular classes of bacteria. Microbes can also regulate signaling pathways in immune cells that result in the production of antimicrobial factors by mammalian cells, effectively resulting in remodeling of intestinal communities and prevention or treatment of infections.

Gastrointestinal infections are a major cause of morbidity and mortality worldwide. Studies conducted in 2006 found that, globally, severe diarrhea and dehydration are responsible each year for the death of 1,575,000 children under the age of five. This represents 15% of the 10.5 million deaths per year of children in this age group [1]. According to recent estimates, acute gastroenteritis causes as many as 770,000 hospitalizations per year in the United States [2]. Enteric pathogens include viruses (rotaviruses, noroviruses) and bacteria such as different strains of pathogenic *Escherichia coli*, toxigenic *Clostridium difficile*, *Campylobacter jejuni*, and *Vibrio cholerae*. These pathogens produce different types of toxins that can cause severe or life-threatening dehydration and diarrhea. Despite medical advances in diagnosis and treatment, the percent and number of hospitalized pediatric patients less than 5 years of age with severe rotavirus infection significantly increased when a recent time period (2001–2003) was compared to an earlier time period (1993–1995) [3]. In addition to the typical pattern of acute gastroenteritis, infectious agents such as enteropathogenic *E. coli* (EPEC) may cause persistent, chronic diarrhea in children lasting longer than 1 week [4]. Such persistent infections may increase the risk of dehydration and long-term morbidities. Importantly, the relative contributions of EPEC and other bacterial pathogens to disease

remains controversial to some extent. A recent study highlighted that increased relative risk of gastrointestinal disease in children was only demonstrable for enteric viruses [5].

Recent studies have highlighted long-term morbidities associated with gastroenteritis. Early childhood diarrhea predisposes children to lasting disabilities, including impaired fitness, stunted growth, and impaired cognition and school performance [6]. Along with this data, new research on maternal and child undernutrition reported in *The Lancet* in January 2008 links poor nutrition with an increased risk for enteric infections in children. Furthermore, irritable bowel syndrome (IBS), a costly and difficult to treat condition that affects 20% of the United States population [7], has medical costs of up to $30 billion per year, excluding prescription and over-the-counter drug costs [8]. IBS is precipitated by an episode of acute gastroenteritis in up to 30% of all cases in prior studies [9]. Therefore, preventing or treating acute gastroenteritis before long-term sequelae develop would drastically reduce hospitalizations, disability-adjusted life years, and both direct and indirect medical costs.

Accurate diagnosis of acute gastroenteritis is an ongoing challenge even in sophisticated academic medical centers. In a pediatric patient population exceeding 4,700 children, less than 50% of stool samples that underwent complete microbiologic evaluation yielded a specific diagnosis [10]. Enteric viruses represented the predominant etiologic agents in acute gastroenteritis in children less than 3 years of age, and bacteria caused the majority of cases of acute gastroenteritis in children older than 3 years of age [10]. The diagnostic challenges with enteric viruses include the relative paucity of stool-based molecular or viral antigen tests and the inability to readily culture most enteric viruses. Bacterial pathogens may be difficult to identify (such as most strains of disease-causing *E. coli*) because of the lack of specific assays for these infections. The relative insensitivity of stool-based toxin assays for the detection of toxigenic *C. difficile* precludes accurate diagnosis. In a children's hospital setting, combination toxin antigen testing yielded sensitivity below 40% in pediatric patients (J. Versalovic, unpublished data). The introduction of new molecular assays for real-time PCR detection of toxin genes directly in stool has markedly improved the ability to diagnose antimicrobial-associated diarrhea and colitis due to toxigenic *C. difficile* [11]. In addition, approximately 15–25% of cases of antimicrobial-associated diarrhea are caused by *C. difficile*. The prevalence of antimicrobial-associated diarrhea and gastrointestinal disease highlights the importance of alternatives to antibiotic strategies for treatment. Furthermore, antibiotics have limited utility for the treatment of gastroenteritis in general. Antimicrobial agents are not generally recommended as prevention strategies because of the problems of antibiotic resistance and antimicrobial-associated disease. Thus, instead of suppressing bacterial populations with antibiotics, can probiotics be used to remodel or shift microbial communities to a healthy state [12]?

2. PROBIOTICS

2.1. The need for mechanistic details of probiotic action

The use of probiotics to prevent and treat a wide variety of conditions has gained favor in the past decade. This is in part due to a need to find alternatives to traditional therapies such as antibiotics as well as the lack of good treatments for GI ailments. While there are increasing reports of the efficacy of probiotics in the treatment of diseases such as pouchitis [13, 14], diarrhea [15–17], and irritable bowel syndrome [18], the scientific basis for the use of probiotics is just beginning to be understood. We will focus on the potential applications for probiotics in the treatment of diarrheal disease. Several examples will highlight how probiotics may be selected for and utilized against pathogens causing gastroenteritis.

The concept of using probiotic microorganisms to prevent and treat a variety of human ailments has been around for more than 100 years [19]. With the rise in the number of multidrug resistant pathogens and the recognition of the role that the human microbiota plays in health and disease, a recent expansion in the interest in probiotics has been generated. This phenomenon is apparent in both the numbers of probiotic products being marketed to consumers as well as the increased amount of scientific research occurring in probiotics. Although many of the mechanisms by which probiotics benefit human beings remain unclear, probiotic bacteria are being utilized more commonly to treat specific diseases.

Several definitions of what constitutes a "probiotic" in the literature have been formulated. For this review, we use the definition derived in 2001 by the Food and Agricultural Organization (FAO) and the World Health Organization (WHO)—"Probiotics are live microorganisms which when administered in adequate amount confer a health benefit on the host." [20]. This definition is the currently accepted definition by the International Scientific Association for Probiotics and Prebiotics (ISAPP) (http://www.isapp.net/).

2.2. Antipathogenic activities

Perhaps the most important scientific question regarding the use of probiotics in medicine is the identification of mechanisms by which probiotics impact human health. Several mechanisms have been implicated but most have not been experimentally proven (Figure 1). Here, we discuss possible mechanisms that are relevant for the treatment of diarrheal diseases. We will highlight research examples that support these putative mechanisms whenever possible.

2.3. Stimulation of host antimicrobial defenses

Many probiotics have been shown to produce antipathogenic compounds ranging from small molecules to bioactive antimicrobial peptides. Most of these studies have focused on the in vitro susceptibility of pathogens to products secreted by probiotic bacteria. In most cases, the ability of an antimicrobial compound secreted by a probiotic organism to inhibit the growth of a pathogen in vivo has not been

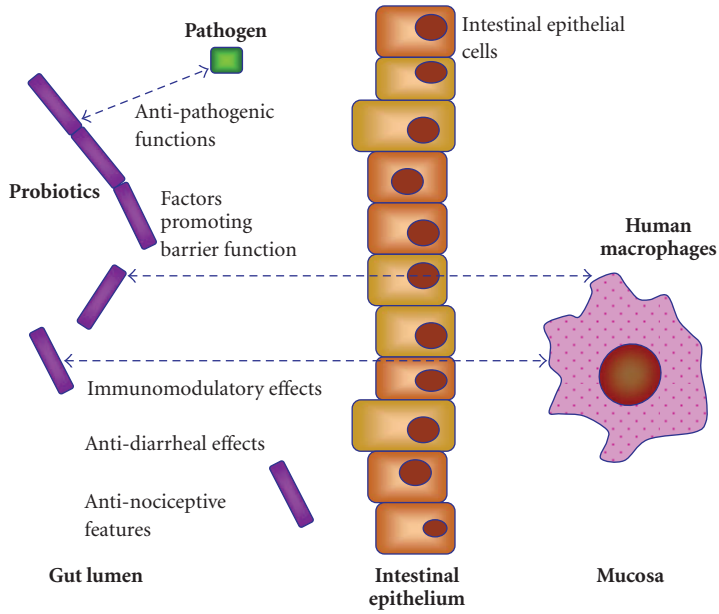

Figure 1: *Probiotics and Beneficial Effects in the Intestine*. Depiction of the interactions between beneficial bacteria (left side), their secreted factors, pathogens, and the intestinal mucosa (right side). Potential beneficial effects of probiotics are listed. Only two host cell types are shown, intestinal epithelial cells and macrophages although other cell types including dendritic cells, lymphocytes, myofibroblasts, and neutrophils comprise the intestinal mucosa. The arrows indicate the release and possible distribution of secreted factors derived from probiotics.

demonstrated. Conceptually, an antimicrobial compound produced by an organism would need to be produced at a high enough level and in the right location in the intestinal tract to exert a strong effect on a pathogen in vivo.

An elegant proof of principle for direct action of a probiotic-produced antimicrobial against a pathogen was recently reported by Corr et al. who demonstrated that production of the bacteriocin Abp118 by *Lactobacillus salivarius* was sufficient to protect mice from disease by infection with *Listeria monocytogenes* [21]. To prove the action of the bacteriocin was directly responsible for the protection of the mice, they generated a *L. salivarius* strain that was unable to produce Abp118 and showed that this mutant was incapable of protecting against *L. monocytogenes* infection. Notably, they were able to express a gene that confers immunity to the Abp118 bacteriocin within *L. monocytogenes* and showed that this strain was now resistant to the probiotic effect of *L. salivarius* within the mouse. This study provided clear evidence that a probiotic-derived bacteriocin could function directly on a pathogen in vivo.

2.4. Pathogen exclusion via indirect mechanisms

In addition to producing antimicrobial compounds that act directly on pathogens, probiotics may stimulate host antimicrobial defense pathways. The intestinal tract has a number of mechanisms for resisting the effects of pathogens including the production of defensins [22]. Defensins are cationic antimicrobial peptides that are produced in a number of cell types including Paneth cells in the crypts of the small intestine and intestinal epithelial cells. A deficiency in alpha-defensin production has been correlated with ileal Crohn's disease [23, 24]. Tissue samples from patients with Crohn's disease showed a lower level of alpha-defensin production and extracts from these samples exhibited a reduced ability to inhibit bacterial growth in vitro. Moreover, some pathogenic bacteria have evolved mechanisms to inhibit the production or mechanism of action of defensins (reviewed in [25]).

Probiotics may act to stimulate defensin activity via at least two mechanisms. First, probiotics may stimulate the synthesis of defensin expression. This has been demonstrated for human beta defensin 2 (hBD-2), whose expression is upregulated by the presence of several probiotic bacteria via the transcription factor NF-κB [26, 27]. The implication is that probiotic strains with this capability would strengthen intestinal defenses by increasing defensin levels. This effect is also observed with certain pathogenic bacteria and thus is not a specific property of probiotic bacteria. Second, many defensins are produced in a propeptide form that must be activated via the action of proteases. One well-characterized example is the activation of the murine defensin cryptdin (an alpha-defensin that is produced by Paneth cells) by the action of matrix metalloprotease 7 (MMP-7) [28]. Mice defective for MMP-7 are more susceptible to killing by *Salmonella*. Evidence indicates that bacteria can stimulate the production of MMP-7 in the intestine [29]. Thus, one mechanism in which probiotics could participate in activating defensins is by stimulating the production of MMPs in the intestinal tract. Alternatively, probiotics could

produce proteases that themselves activate defensins in the intestinal lumen. Although there is no evidence yet to support this mechanism, a subset of lactobacilli and streptococci encode MMP-like proteins in their genomes (R. Britton, unpublished observation). These MMPs are not found in any other bacteria and thus it will be interesting to determine what effect they have on host cell function.

2.5. Immunomodulation

Rather than directly inhibiting the growth or viability of the pathogen, probiotics may compete for an ecological niche or, otherwise, create conditions that are unfavorable for the pathogen to take hold in the intestinal tract. There are many possible mechanisms for how pathogen exclusion may take place. First, several probiotics have been demonstrated to alter the ability of pathogens to adhere to or invade colonic epithelial cells in vitro, for example, see [30, 31]. Second, probiotics could sequester essential nutrients from invading pathogens and impair their colonization ability. Third, probiotics may alter the gene expression program of pathogens in such a way as to inhibit the expression of virulence functions [32]. Lastly, probiotics may create an unfavorable environment for pathogen colonization by altering pH, the mucus layer, and other factors in the local surroundings. It is important to note that although many of these possible effects have been demonstrated in vitro, the ability of probiotics to exclude pathogens in vivo remains to be proven.

2.6. Enhancing intestinal barrier function

Probiotics may have strain-dependent effects on the immune system. Different strains representing different *Lactobacillus* species demonstrated contrasting effects with respect to proinflammatory cytokine production by murine bone marrow-derived dendritic cells [33]. Specific probiotic strains counteracted the immunostimulatory effects of other strains so that probiotics have the potential to yield additive or antagonistic results. Interestingly, in this study, the anti-inflammatory cytokine IL-10 was maintained at similar levels [31]. Different probiotic *Lactobacillus* strains of the same species may also yield contrasting effects with respect to immunomodulation. Human breast milk-derived *Lactobacillus reuteri* strains either stimulated the key proinflammatory cytokine, human tumor necrosis factor (TNF), or suppressed its production by human myeloid cells [34]. The mechanisms of action may be due, not surprisingly, to contrasting effects on key signaling pathways in mammalian cells. Probiotic strains such as *Lactobacillus rhamnosus* GG (LGG) may activate NF-κB and the signal transducer and activator of transcription (STAT) signaling pathways in human macrophages [35]. In contrast, probiotic *Lactobacillus* strains may suppress NF-κB signaling [36, 37] or MAP kinase-/c-Jun-mediated signaling [34]. Stimulation of key signaling pathways and enhancement of proinflammatory cytokine production may be important to "prime" the immune system for defense against gastrointestinal infections. Conversely, suppression of immune signaling may be an important mechanism to promote homeostasis and tolerance to microbial communities with many potential antigens, and these immunosuppressive functions may promote healing or resolution of infections.

2.7. Why understanding mechanisms is important?

The disruption of epithelial barrier function and loss of tight junction formation in the intestinal epithelium may contribute to pathophysiology and diarrheal symptoms observed during infection with certain pathogens [38, 39]. Loss of tight junctions can lead to increased paracellular transport that can result in fluid loss and pathogen invasion of the submucosa. Pathogens may secrete factors such as enterotoxins that may promote excessive apoptosis or necrosis of intestinal epithelial cells, thereby disrupting the intestinal barrier. Enteric pathogens may also cause effacing lesions at the mucosal surface due to direct adherence with intestinal epithelial cells (e.g., EPEC). In contrast, probiotics have been reported to promote tight junction formation and intestinal barrier function [40, 41]. Although the mechanisms of promoting barrier integrity are not well understood, probiotics may counteract the disruption of the intestinal epithelial barrier despite the presence of pathogens. Probiotics may also suppress toxin production or interfere with the abilities of specific pathogens to adhere directly to the intestinal surface. As a result, pathogens may have a diminished ability to disrupt intestinal barrier function.

2.8. Important considerations for the use of probiotics: strain selection and microbial physiology

An important challenge in the field of probiotics is the identification of genes and mechanisms responsible for the beneficial functions exerted by these microbes. Successful identification of mechanistic details for how probiotics function will have at least three important benefits. First, understanding mechanisms of action will provide a scientific basis for the beneficial effects provided by specific microbes. These breakthrough investigations will help move probiotics from the status of dietary supplements to therapeutics. Second, understanding mechanisms of probiosis and the gene products produced by probiotics will allow for the identification of more potent probiotics or the development of bioengineered therapeutics. As an example, the anti-inflammatory cytokine IL-10 was postulated to be a potential therapeutic for the treatment of inflammatory bowel disease. To test this hypothesis, a strain of *Lactococcus lactis* engineered to produce and secrete IL-10 was constructed and demonstrated to reduce colitis in a murine model [42]. Early clinical trials in patients with inflammatory bowel disease indicate some relief from symptoms when treated with the IL-10 overproducing strain. Third, the identification of gene products that are responsible for ameliorating disease will allow researchers, industry, and clinicians to follow the production of these products as important biomarkers during probiotic preparation. As discussed below, the physiological state of microbes can be crucial to the functions of probiotics. Thus, it will be important to be able to follow the

production of important bioactive molecules when culturing and processing probiotics for applications in animals and humans.

2.9. Probiotics and diarrhea

Probiotics are considered to be living or viable microorganisms by definition. Unlike small molecules that are stable entities, probiotics are dynamic microorganisms and will change gene expression patterns when exposed to different environmental conditions. This reality has two important implications for those who choose to use these organisms to combat human or animal diseases. First, probiosis is a strain-specific phenomenon. As defining a bacterial species is challenging in this age of full genome sequencing, it is clear that probiotic effects observed in vitro and in vivo are strain specific. For example, modulation of TNF production by strains of *Lactobacillus reuteri* identified strains that were immunostimulatory, immunoneutral, and immunosuppressive for TNF production [34, 43]. These findings highlight the strain-specific nature of probiotic effects exerted by bacteria. Thus, it is important for research groups and industry to be cautious with strain handling and tracking so that inclusion of correct strains is verified prior to administration in clinical trials.

The second key point is that the physiology of the probiotic strain is an important consideration. Being live microorganisms, the proteins and secondary metabolites that are being produced will change depending on growth phase. This feature raises a number of important issues for the stability and efficacy of probiotic strains. First, probiotics are subjected to numerous environmental stresses during production and after ingestion by the host. Most notably, probiotics used to treat intestinal ailments or whose mode of action is thought to be exerted in the intestinal tract must be able to survive both acid and bile stress during transit through the gut. The physiological state of the microbe is an important characteristic that determines whether cells will be susceptible to different types of environmental stress [44, 45]. For example, exponentially growing cells of *L. reuteri* are much more susceptible to killing by bile salts than cells in stationary phase [45]. Thus, it is important to consider the physiological state of the cells in terms of stress adaptation not only for survival in the host but also during production. Second, the expression of bioactive molecules, which are most often responsible for the health benefits exerted by probiotics, is often growth phase-dependent. For example, our groups have been investigating the production of immunomodulatory compounds and antimicrobial agents by strains of *L. reuteri*. In both cases, these compounds are more highly expressed in the entry into and during stationary phase (unpublished observation).

3. PROBIOTICS AND THE PREVENTION AND TREATMENT OF GASTROENTERITIS—EXAMPLES

Commensal-derived probiotic bacteria have been implicated as therapy for a range of digestive diseases, including antibiotic-associated colitis, *Helicobacter pylori* gastritis, and traveler's diarrhea [46]. Probiotic formulations may include single strains or combinations of strains. *L. reuteri* is indigenous to the human gastrointestinal tract, is widely present in mammals, and has never been shown to cause disease. In human trials, probiotic treatment with *L. reuteri* in small children with rotaviral gastroenteritis reduced the duration of disease and facilitated patient recovery [15, 16], while in another study, it prevented diarrhea in infants [17]. Despite the promising data from clinical trials, the primary molecular mechanisms underlying the antipathogenic properties of *L. reuteri* remain unknown.

Probiotics may be effective for the prevention or treatment of infectious gastroenteritis. In the context of disease prevention, several studies with different probiotic strains have documented that these bacteria may reduce the incidence of acute diarrhea by 15–75% depending on the study [17, 47–50]. Although the relative impacts on disease incidence vary depending on the specific probiotic strain and patient population, consistent benefits for disease prevention have been demonstrated in multiple clinical studies. In one disease prevention study [49], supplementation with *Bifidobacterium lactis* significantly reduced the incidence of acute diarrhea and rotavirus shedding in infants. Studies that examined potential benefits of probiotics for preventing antimicrobial-associated diarrhea have yielded mixed results [51–54]. One prevention study reported a reduction in incidence of antimicrobial-associated diarrhea in infants by 48% [52].

Probiotics may also be incorporated in treatment regimens for infectious gastroenteritis. Several meta-analyses of numerous clinical trials with different probiotics documented reductions in disease course of gastroenteritis that ranged from 17 to 30 hours [49, 50, 55]. Examined another way, meta-analyses of probiotics used in clinical trials of gastroenteritis noted significant reductions of incidence of diarrhea lasting longer than 3 days (prolonged diarrhea). The incidence of prolonged diarrhea was diminished by 30% or 60%, respectively, depending on the study [50, 56] (summarized in [55]). The probiotic agent, LGG, contributed to a significant reduction in rotavirus diarrhea by 3 days of treatment when administered to children as part of oral rehydration therapy [57]. Recent data compilations of a large series of probiotics trials by the Cochrane Database of Systematic Reviews (http://www.cochrane.org/) have yielded promising conclusions. As of 2008, probiotics appear to be effective for preventing acute gastroenteritis in children and may reduce duration of acute disease. Additionally, probiotics are promising agents for preventing and treating antimicrobial-associated diarrhea, although intention-to-treat analyses have not demonstrated benefits.

3.1. Clostridium difficile and antibiotic-associated diarrhea

In what follows, we highlight some possible mechanisms by which probiotics can be used to ameliorate gastroenteritis. Because a number of infectious agents cause diarrhea, colitis, and gastroenteritis, we will only focus on a few examples

with the idea that many of the mechanisms discussed can be extended to other bacterial or viral causes of diarrhea.

3.1.1. The potential role of probiotics in treating CDAD

An estimated 500,000–3,000,000 cases of *Clostridium difficile*-associated diarrhea (CDAD) occur annually with related health care costs exceeding $1 billion per year [58–60]. CDAD occurs primarily in patients that have undergone antibiotic therapy in a health care setting, indicating that alterations in the intestinal microbiota are important for the initiation of CDAD. In a small but increasing number of cases, more severe complications will occur including pseudomembranous colitis and toxic megacolon. Moreover, the emergence of metronidazole-resistant strains of *C. difficile* has diminished the efficacy of metronidazole, and vancomycin- and metronidazole-induced cecitis reinforces the need for new therapies for the treatment and prevention of CDAD [61, 62].

Approximately 10–40% of patients treated for an initial bout of CDAD will show recurrent disease, often with multiple episodes [63]. Such recurrences are often refractory to existing therapies including antibiotic therapy. Patients with recurrent CDAD had a marked decrease in the diversity of organisms in their fecal microbiota while patients that were free of recurrent disease had a normal microbiota [64]. Thus, therapies that restore a normal microbiota or suppress *C. difficile* growth while allowing the repopulation of the intestine with a favorable microbiota may be important to resolve infections and maintain intestinal health.

3.1.2. Eradication of C. difficile through the production of antimicrobial compounds

Probiotic organisms have been used to treat recurrent *C. difficile* in the past and in a few cases have showed a modest effect in ameliorating recurrent disease [63]. This application has been somewhat controversial and at this time the use of probiotics in ameliorating CDAD is not recommended [65]. However, the organisms tested were not specifically isolated for the treatment of CDAD and, therefore, may have not been the appropriate strains to be used to prevent recurrent CDAD. In what follows, we outline potential mechanisms in which carefully selected or engineered probiotics could be used in the treatment of *C. difficile* and the eradication of this pathogen.

3.1.3. Competitive exclusion of C. difficile using probiotics

CDAD is currently treated by the use of antimicrobial agents that are effective against *C. difficile*, most often vancomycin or metranidazole. Because these drugs are broad-spectrum antibiotics, they likely play a role in recurrent disease by suppressing the normal intestinal microbiota. Using antimicrobial compounds that target *C. difficile* while allowing restoration of resident organisms would be one possible mechanism to prevent recurrent CDAD.

3.1.4. Probiotics and C. difficile spore germination

As mentioned above, CDAD is usually an infection that is acquired in the hospital or other health care setting.

Therefore, a probiotic that could competitively exclude *C. difficile* could be administered prior to entry into the hospital. Unfortunately, little is known about how and where *C. difficile* colonizes the intestine. Once this information is known, strategies for blocking colonization with probiotics can be developed.

Nonetheless, a promising probiotic approach using nontoxigenic *C. difficile* has been described. Using a hamster model of *C. difficile* infection, Gerding et al. demonstrated a protective effect of populating the hamster with strains of *C. difficile* that are unable to produce toxin prior to challenge with a virulent toxin-producing strain [66]. Colonization of the intestinal tract by the nontoxigenic strain appeared to be required for protection. Currently, this probiotic approach is under investigation for use in humans (http://www.viropharma.com/).

3.2. Enterohemorrhagic E. coli

A likely contributor to the difficulty in eradicating *C. difficile* from the intestine is the ability of the organism to develop stress-resistant spores. The identification of probiotic strains that can prevent either spore formation or the germination of spores in the intestinal tract provides a promising avenue to combat CDAD. Recent work on spore germination has provided in vitro assays in which inhibitory activities of probiotics can be tested [67].

Germination of spores in the laboratory requires the presence of bile acids, with taurocholate and cholate demonstrating the best activity [67]. Thus, bile acids could play a role in signaling to *C. difficile* that spores are in the correct location of the gut to germinate. Sorg and Sonenshein have recently proposed a mechanism by which the reduction in the intestinal microbiota could lead to efficient spore germination and overgrowth of *C. difficile* [67]. They found that the bile acid deoxycholate (DOC) was able to induce spore germination but that subsequent growth was inhibited due to toxic effects of DOC on vegetative *C. difficile*. Their work suggests a model in which a reduction in the concentration of DOC in the intestine, due to the disruption of the normal microbiota, removes this key inhibitor of *C. difficile* growth. DOC is a secondary bile acid produced from dehydroxylation of cholate by the enzyme 7α-dehydroxylase, an activity that is produced by members of the intestinal microbiota. While it is unclear whether or not antibiotic therapy reduces the level of DOC in the intestine, it is tempting to speculate that providing probiotic bacteria capable of producing 7α-dehydroxylase may prevent intestinal overgrowth by *C. difficile* while the normal microbiota is being reestablished.

3.2.1. Toxin sequestration and removal

Enterohemorrhagic *E. coli* (EHEC) infections cause sporadic outbreaks of hemorrhagic colitis throughout the world (~100,000 cases per year in the United States) [68]. Most

infections result in the development of bloody diarrhea but a subset (~5–10%) of EHEC patients (mostly children) will develop the life-threatening condition hemolytic uremic syndrome (HUS) [69, 70]. HUS is the leading cause of kidney failure in children. EHEC, which likely evolved from an EPEC strain [71], also produces attaching and effacing lesions on host epithelial cells and reduces intestinal epithelial barrier function. In addition, EHEC strains are characterized by the expression of Shiga toxin (Stx) genes, and thus they can be labeled as Shiga-toxin-producing *E. coli* (STEC). Currently, only supportive therapy for EHEC infection is available since antibiotic therapy may increase the risk of developing HUS, and therefore, novel therapies must be developed. One promising alternative therapeutic may be the use of probiotics to treat EHEC infections.

3.2.2. *Inhibition of toxin production by EHEC—identification of strains that repress the lytic functions of lambda*

Shiga toxins are ribosome-inactivating proteins that inhibit protein synthesis by removing a specific adenine residue from the 28S rRNA of the large ribosomal subunit [72]. Shiga toxin is required for the development of HUS and recent work has indicated that EHEC strains mutated for Shiga toxin production fail to cause disease in a germfree mouse model [73]. Indeed, injection of Shiga toxin with LPS directly into mice is sufficient to generate a HUS-like disease in the kidneys of mice [74]. Therefore, Shiga toxin is an important mediator of HUS and therapies aimed at neutralizing its activity are expected to reduce or eliminate this life-threatening complication although current attempts at Shiga toxin neutralization have been unsuccessful [75].

As a possible mechanism for treating EHEC disease and reducing the incidence of HUS cases, Paton et al. have generated "designer probiotics" in which the oligosaccharide receptor (Gb_3) for Stx is expressed on the cell surface of an *E. coli* strain [76–78]. This probiotic strain was shown to be capable of neutralizing Stx in vitro. As a proof-of-concept, mice that were challenged with a STEC strain were protected by administration of the probiotic expressing the Gb_3 receptor [79]. The protective effect was observed even when the strains were formalin-killed prior to use, supporting the hypothesis that toxin sequestration and removal was the mechanism by which the mice were protected. Similar results have been obtained using bacteria-expressing receptors for toxins produced by other diarrheal pathogens including enterotoxigenic *E. coli* (most common cause of traveler's diarrhea) and *Vibrio cholerae*.

3.2.3. *Inhibition of pathogen adherence and strengthening of intestinal barrier functions*

Stx genes are carried on lambdoid prophages and are usually located in a late transcribed region of the virus, near the lytic genes [80]. Since no mechanism for toxin secretion has been identified, the location of Stx near the lytic genes suggests that phage activation and cell lysis are responsible for Stx production and release. This genetic juxtaposition suggests that therapeutics that suppress the lytic decision of lambda in vivo would greatly reduce or eliminate complications caused by systemic release of Stx.

3.3. *Rotavirus*

A key interaction of EHEC, as well as EPEC, with the intestinal epithelium is the formation of attaching and effacing lesions on the surface of the epithelium [81]. This interaction is brought about by factors secreted directly from the bacterium into the host cell, where a redistribution of the actin cytoskeleton occurs. EHEC and EPEC infection also induces a loss of tight junction formation and reduction of the intestinal epithelial barrier by inducing the rearrangement of key tight junction proteins including occludin [82, 83]. Therapies that would either disrupt this interaction of EHEC/EPEC with the intestinal epithelium or inhibit the loss of barrier function should ameliorate disease.

Probiotics have shown some success inhibiting adhesion, A/E lesion formation and enhancing barrier function in response to EHEC infection in vitro. Johnson-Henry et al. tested the ability of *Lactobacillus rhamnosus* GG to prevent loss of barrier integrity and formation of A/E lesions induced by EHEC infection of cell culture in vitro [40]. They found that pretreatment of intestinal epithelial cells in vitro with LGG was sufficient to reduce the number of A/E lesions and to prevent loss of barrier function as measured by transepithelial resistance, localization of tight junction proteins, and barrier permeability assays. Importantly, live LGG was required for these effects as heat-killed bacteria were not effective in preventing EHEC effects on epithelial cells.

Enteric viruses including noroviruses and rotavirus represent major causes of gastroenteritis, especially in young children. Rotavirus infection results in acute gastroenteritis with accompanying dehydration and vomiting mainly in children 3–24 months of age. Human rotavirus primarily infects intestinal epithelial cells of the distal small intestine, resulting in enterotoxin-mediated damage to intestinal barrier function. Recent studies indicate that probiotics may reduce the duration and ameliorate disease due to rotavirus infection ([84]; G. Preidis and J. Versalovic, unpublished data). Probiotics promoted intestinal immunoglobulin production and appeared to reduce the severity of intestinal lesions due to rotavirus infection in a mouse model. These findings and related investigations suggest that probiotics may diminish the severity and duration of gastrointestinal infections by mechanisms independent of direct pathogen antagonism. Probiotics may also promote healing and homeostasis by modulating cytokine production and facilitating intestinal barrier function.

4. CONCLUDING REMARKS

Probiotics may provide an important strategy for the prevention and treatment of gastrointestinal infections. Specific bacteria derived from human microbial communities may have key features that establish these microbes as primary candidates for probiotic therapies. These beneficial microbes

may have different effects within the host such as prevention of pathogen proliferation and function. Probiotics may also stimulate the host's immune function and mucosal barrier integrity. By working via different mechanisms of probiosis, probiotics may yield effects at different steps in the process. Probiotics may prevent disease from occurring when administered prophylactically. Probiotics may also suppress or diminish severity or duration of disease in the context of treatment. As our knowledge of the human microbiome advances, rational selection of probiotics based on known mechanisms of action and mechanisms of disease will facilitate optimization of strategies in therapeutic microbiology. Ultimately, we expect that probiotics will help to promote stable, diverse, and beneficial microbial communities that enhance human health and prevent disease.

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Review Article

Probiotic Bacteria Influence the Composition and Function of the Intestinal Microbiota

Paul W. O'Toole[1] and Jakki C. Cooney[2]

[1] *Department of Microbiology and Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland*
[2] *Department of Life Sciences and Materials and Surface Sciences Institute, University of Limerick, Limerick, Ireland*

Correspondence should be addressed to Paul W. O'Toole, pwotoole@ucc.ie

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Probiotics have a range of proposed health benefits for the consumer, which may include modulating the levels of beneficial elements in the microbiota. Recent investigations using molecular approaches have revealed a human intestinal microbiota comprising over 1000 phylotypes. Mechanisms whereby probiotics impact on the intestinal microbiota include competition for substrates, direct antagonism by inhibitory substances, competitive exclusion, and potentially host-mediated effects such as improved barrier function and altered immune response. We now have the microbial inventories and genetic blueprints to begin tackling intestinal microbial ecology at an unprecedented level of detail, aided by the understanding that dietary components may be utilized differentially by individual phylotypes. Controlled intervention studies in humans, utilizing latest molecular technologies, are required to consolidate evidence for bacterial species that impact on the microbiota. Mechanistic insights should be provided by metabolomics and other analytical techniques for small molecules. Rigorous characterization of interactions between the diet, microbiota, and probiotic bacteria will provide new opportunities for modulating the microbiota towards improving human health.

1. INTRODUCTORY REMARKS

The history of microbiological research has been dominated by investigations of the agents of human infectious disease. Motivated by the desire to culture, characterize, and understand the pathogenicity mechanisms of these organisms, several centuries of microbiological research culminated in a broad range of antimicrobial therapies, vaccines, and immunizations. In more recent years, similar analytical methodologies have been applied to facilitate exploitation of bacteria for industrial applications. Two related branches of microbiology—environmental microbiology, and the study of intestinal commensals (a branch of the first in purest terms)—lagged behind until relatively recently. From the mid 1990s, a range of techniques allowed environmental microbiologists to indentify soil microorganisms *in situ*, without resorting to culture, based upon ribosomal small subunit RNA gene probes. A natural extension of this approach was to sequence large numbers of cloned ribosomal RNA gene amplicons, yielding catalogs of all the organisms (the microbiota) present in complex samples. Latterly the field of metagenomics has provided technical approaches to sequence large fractions of the entire microbial DNA present in an ecological system. Coupled with the application of molecular tools for studying commensal bacteria, many of which were originally developed for studying pathogens, there is now an exciting nexus between technologies and research foci whereby commensal bacteria may be studied in the context of intestinal ecosystems. This review will summarize what is known about the effect of introducing probiotic bacteria on the composition and activities of the microbiota, with an emphasis on recent studies using culture-independent methods. The likely mechanisms whereby commensals exert their influence are discussed, and directions for future research are outlined.

2. THE CONCEPTS OF PROBIOTICS AND PREBIOTICS

The notion that certain intestinal microorganisms might benefit the host derives historically from suggestions by Metchnikoff and others that putrefying bacteria that contribute to toxification and aging could be deliberately

Table 1: Beneficial properties reported for probiotic bacteria.

Host benefit	Microbial trait implicated	Reference[1]
Immune modulation		
Stimulation of immunity	Enhance T-cell numbers and activity levels	[13]
Dampening of inflammation	Promote anti-inflammatory cytokine production	[14]
Pathogen burden reduction	Competitive exclusion	[15]
	Direct antagonism	[16, 17]
	Uncharacterised	[18, 19]
Improved gut barrier function	Promote gut barrier integrity	[20]
Reduced cancer risk	Detoxification of carcinogenic metabolites	[21]
Reduced atopic allergy symptoms	Suppression of hypersensitivity	[22]
Reduced cardiovascular disease risk	Cholesterol reduction by deconjugation of bile salts	[23, 24]
	Production of anti-hypertensive peptides	[25]
Alleviation of dietary intolerance	Catabolism of dietary ingredients	[26]
Enhanced nutrient value	Vitamin and co-factor production	[27]
Alleviation of IBS[2] symptoms	Not defined	[28, 29]

[1]Sample reference for each trait. See main text for review references,
[2]Irritable bowel syndrome.

replaced by fermentative organisms [1, 2]. In this context, some of the fermentative bacteria, Metchnikoff was referring to, are what we now consider as probiotic. Probiotic bacteria are live microorganisms which when administered in adequate amount confer a health benefit on the host [3, 4]. Many microorganisms that are considered probiotic have been traditionally used to preserve food products by fermentation, and are present in the food in varying numbers, along with their fermentation end products and other metabolites. Thus another operational definition of the term probiotic requires the organism in question to be "consumed in adequate amounts" to confer a benefit [5]. The host benefits that have been attributed to consumption of probiotic microorganisms are diverse (reviewed in [3, 6–8]; some major examples are listed in Table 1), and have been substantiated to different degrees. Probiotic bacteria are now included in a wide range of consumer formulations including yoghurts, drinks, capsules, and dietary supplements, and they represent a significant element in the modern functional foods market. Organisms used as probiotic agents are frequently members of the genera *Lactobacillus* or *Bifidobacterium*, but *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces boulardii,* and *Enterococcus faecium* are also employed, among others [9]. Thus, an organism employed as a probiotic agent may not necessarily be part of what is considered the "normal microbiota." Tannock distinguishes between allochthonous and autochthonous species [10, 11]. Autochthonous means bacteria both present and replicating *in situ* in the human GI tract, as distinct from transiently passing through (allochthonous). Bacteria administered as probiotic agents are not necessarily autochthonous to the consuming animal, and indeed some *Lactobacillus* species may only be autochthonous for certain human individual subjects, and possibly not the majority of subjects (see below). Rate of growth of allochthonous lactobacilli may be a critically limiting step preventing their establishment [12]. With regard to developing probiotic strains for exploitation, it may prove easier to identify beneficial traits in species that are autochthonous to the human consumer, as consumer acceptance is likely to be easier if the probiotic ingredient in a functional food (a food product with benefit to the consumer over and above inherent nutrition) was first cultured from humans.

Related to the consumption of probiotic agents is the notion of dietary adjustment to stimulate bacterial growth. A prebiotic compound is defined as "a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improves host health" [30, 31]. It follows from this definition that the bacteria capable of metabolizing prebiotics should be restricted to a small number of beneficial species or strains (reviewed in [32]). In practice, prebiotic compounds must also be refractory to host digestive processes, and the combined catabolic activities of bacteria higher up in the gastrointestinal tract, so that prebiotic compounds are often oligosaccharides towards which probiotic bacteria produce specific hydrolases [33]. Prebiotics are commonly found in, or extracted from, plant material including fruits, cereal, and vegetables, but are also present in human milk and colostrum [6]. The best characterized prebiotics include inulin, fructooligosaccharide, galactooligosaccharide, xylooligosaccharide, isomaltooligosaccharide, and lactulose (reviewed in [32]). Unravelling the health benefits of prebiotics is a challenging task, because these compounds have parallel direct effects on the host, and potentially on multiple members of the microbiota. For example, β-glucans are unbranched polysaccharides with (1-4) and (1-3)-linked β-D glucopyranosyl units, that are recognized as important dietary ingredients (reviewed in [34]). β-glucans are components of plant cell walls, and are abundant in the endosperm of cereals such as barley and oatmeal. Consumption of β-glucans has attendant health

benefits that are recognized by health and regulatory bodies in several jurisdictions including the US [35]. These benefits include lowering of blood cholesterol and lipoprotein [36], lowering of postprandial glucose and insulin responses [37], and enhancement of antitumor monoclonal antibodies [38]. Supplementation of mammalian diet with β-glucan, or modification by prehydrolysis of *in vitro* bacterial growth medium, leads to increased numbers and proportions of lactobacilli or bifidobacteria [39–41]. Furthermore, β-glucooligomers promoted the growth of *L. rhamnosus* GG [40]. Barley supplementation of rat diet [39] led to an increase in *Lactobacillus* numbers, a decrease in *Bacteroides* and coliforms, and an increase in the production of butyrate. Butyrate is an important energy source, signalling metabolite, proliferation stimulus for normal colonic epithelial cells, and anti-proliferative signal for neoplastic colonocytes [42, 43], suggesting a potential direct benefit from dietary ingredients or prebiotics that promote growth of clostridia.

3. THE NORMAL MICROBIOTA OF HUMANS AND ANIMALS

Until recently, the composition of the microbiota was examined by relatively insensitive techniques. Culturing the bacteria was unrepresentative, because a large proportion of the bacteria do not grow on standard laboratory media [44]. Analysis by temperature gradient gel electrophoresis provided one of the earliest insights into the uncharted complexity of the microbiota [45]. Using denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene amplicons, the same group later showed that the colonic mucosal microbiota and faecal microbiota were different, and the colonic mucosal microbiota was likely dependent on host factors [46]. Meanwhile fluorescent hybridization of probes for 16S rRNA genes was being applied to determine species identities, numbers, and proportions of intestinal bacteria [47, 48], exemplified by the studies of Dore, Blaut and colleagues [49, 50]. These analyses highlighted extensive inter-individual variation at phylotypes level (among northern Europeans) [49], and some correlations of microbiota with age, gender, and geographic origin but which varied between countries [50].

Our current understanding of the intestinal microbiota (reviewed in [51, 52]) has been significantly shaped by culture-independent methods, in particular the sequencing of 16S rRNA gene amplicons, either from clone libraries or direct pyrosequencing of the PCR product. A consensus appears to be emerging in the literature of somewhere between 800 and 1000 bacterial phylotypes being present in the healthy human intestine [52]; the evidence for which will be selectively presented here. A relatively small-scale investigation by Benno and colleagues in 2003 revealed an unexpectedly high number of novel phylotypes in 240 partially sequenced 16S rRNA gene amplicons clones derived from six elderly individuals [53]. In a pioneering study, Relman and colleagues applied the 16S rRNA molecular inventory-based approach, at a much larger scale than previously published, on samples from both colonic sites and faeces [54]; strikingly, the majority of the sequences derived corresponded to uncultivated species and novel microorganisms. The human stomach, previously considered sterile except for infections with *Helicobacter pylori*, was revealed by 16S rRNA gene library sequencing to be well populated by bacteria, based on detection of 128 bacterial phylotypes from 23 gastric endoscopy samples [55]. Gill and colleagues [56] sequenced not just 16S rRNA genes, but also randomly cloned bacterial DNA—so-called *metagenomics*, a concept developed for environmental community analysis [57]. Gill et al. showed by this metagenomic approach that the bacteria in the gut significantly expand the metabolic capabilities of the human gut [56]. By generating two to three 454 pyrosequencing runs per mouse cecum, Gordon and colleagues showed the existence of an obesity-associated gut microbiome with increased capacity for energy harvest [58]. Significantly, this balance of the microbiota was borne out when investigating obese humans [59], showing a seminal link between human obesity and changes in the microbiota. Furthermore, the complexity of the microbiota in humans and 59 other mammalian species was shown to be linked to phylogeny (of the mammal) and the composition of the diet [60]. Analysis of the metabolic capability likely conferred by the microbial metagenome recently revealed 237 gene families commonly enriched in adult-type and 136 families in infant-type microbiomes [61]. Thus, any consideration of the effect of probiotics on the intestinal metagenome should ultimately include analysis of the downstream effects upon the host of impacting on this metabolic capability. A more fundamental consideration is that the genera whose members are among the most commonly employed probiotics—*Bifidobacterium* and *Lactobacillus*—are not present in the human gastrointestine at the high levels traditionally expected based on culture-based approaches, being represented by 20 phylotypes (ca. 2%) and 36 phylotypes (ca. 3.6%), respectively [52]. Thus if probiotic bacteria impart health benefits to the host under "natural conditions," that is, in individuals who have normal nonmanipulated numbers of probiotic bacteria, they accomplish this despite being at much lower numbers than are achieved by consumption of probiotic products.

4. PROBIOTICS AND THEIR EFFECT UPON THE MICROBIOTA

There have been relatively few studies which have rigorously characterized the effect upon the whole microbiota of administering probiotic cultures, and until recently, all such studies applied targeted analysis of specific groups of bacteria. In one of the earliest investigations, Tannock and colleagues observed transient and modest fluctuations in lactobacillus and bifidobacterium numbers following consumption of a probiotic *L. rhamnosus* strain DR20 [62]. Lactobacilli and enterococci were detected more frequently (among 10 subjects) and in higher numbers during consumption. Interestingly, the presence of stable populations of lactobacilli before the administration period precluded long-term colonization by the administered probiotic strain [62]. Most subjects ceased shedding the probiotic strain in faeces soon after its consumption stopped, but the *L. rhamnosus* strain remained detectable in faeces of one subject

over 2 months after the test period. These data suggest inter-host variables such as bacterium-host or bacterium-diet interactions.

Probiotics and prebiotics are commonly applied in companion animals and production animals [63, 64], and there have been some studies of effects upon the microbiota. Administration of a cocktail containing lactobacilli, bifidobacteria, enterococci, and pediococci improved weight gain in broiler chickens, which was associated with an increase in numbers of *Bifidobacterium* spp., *Lactobacilli*, and Gram-positive cocci [65]. Administration of a probiotic *Enterococcus faecium* strain reduced *E. faecalis* numbers in the intestines of weanling piglets, but total numbers of *E. faecium* remained unchanged, suggesting that the administered strain had displaced part of a fixed number of niche sites occupied by the same species [66]. Many investigations have been published describing the effects of probiotic bacteria on human pathogens (reviewed in [67]), some of which are normal components of production animal microbiota. Enterobacteriaceae numbers were reduced when a cocktail of two *Lactobacillus* strains was administered to pigs [68], and a five-strain probiotic combination reduced *Salmonella enterica* serovar Typhimurium shedding in pigs [69]. Although data from small animal models for human probiotic strains must be interpreted with caution [70], it was interesting to note from a recent study that administration of *L. casei* and *L. plantarum* affected the diversity of murine intestinal lactobacilli, but not the overall bacterial community structure [71]. There was an increase in the number of lactobacilli related to the *acidophilus* complex in the inoculated mice. These animal models provide an opportunity for determining the effect of probiotic administration on the entire microbiota but must ultimately be repeated in humans if that species is the desired host.

Studies in humans are currently few in number, and are often focused in nature. For example, consumption of a commercial probiotic yoghurt reduced *Clostridium difficile*-related diarrhoea in hospitalized patients, but effects on the broader microbiota were not studied [18]. Alterations in the fecal microbiota have been reported in irritable bowel syndrome (IBS) [72, 73]. However, administration of a multispecies probiotic supplementation which alleviated IBS had negligible effect upon the composition of microbiota as measured by quantitative PCR with group-specific primers [74]. However, this approach may have missed changes in microbial composition within these groups. A follow-up study reported stabilization of the microbiota over time [75], which was related to amelioration of symptomatology that was absent from the placebo control group. Alterations of the human intestinal microbiota have also been reported in inflammatory bowel disease (IBD) [76–78]. Given the clinical impetus to find simple non-medicinal solutions to IBD and IBS, one can anticipate renewed vigor in studies of probiotic bacteria as agents for microbiota modulation in these subjects. Probiotics also appear to be efficacious as adjunct therapy for infectious diarrhea, with a recent meta-analysis revealing reduction in risk and duration of diarrhea [79]. Most of the 23 studies included in this analysis were descriptive rather than investigative of the microbiological aspects, and future determination of the effects on the microbiota wrought by probiotic intervention will be very informative. As recently as 2006, the effect of probiotic administration in humans was still being followed by bacteriological culture, but as concluded by the authors of one such study, there was a clear case for culture-independent molecular methods to be applied instead [80]. Community profiling by DGGE showed that lactulose increased the levels of *Bifidobacterium adolescentis* in subjects consuming the prebiotic lactulose, whereas the probiotic yeast *S. boulardii* did not cause any significant universal changes in DGGE profiles [81].

5. MECHANISMS OF PROBIOTIC IMPACT ON THE MICROBIOTA

Figure 1 shows a schematic overview of the potential mechanisms whereby probiotic micro-organisms might influence the intestinal microbiota. Consumption of probiotic cultures may modulate the microbiota or change its metabolic properties by competition for nutritional substrates. Gordon and colleagues have used transcriptional microarrays to show that introducing a probiotic into the mouse gut changes the way the endogenous microbiota metabolize the diet [82]. When germ-free mice that had been monoassociated with *Bacteroides thetaiotaomicron* were challenged with *Bifidobacterium animalis* or *Lactobacillus casei*, both interventions caused shifts in the gene expression pattern of the *B. thetaiotaomicron* genome [82]. These differentially expressed gene sets (i.e., in response to the two probiotics) did not overlap, emphasizing that different probiotics elicit different responses. However, many of the genes in *B. thetaiotaomicron* whose expression was altered by presence of either probiotic strain were related to expansion of the carbohydrate metabolizing capability of *B. thetaiotaomicron*. Thus, one of the ways in which probiotics can impact upon the composition of the microbiota is apparently by competing with them for substrate availability, and by altering the dynamics of carbohydrate utilization by individual microbiota components. This competition is probably not restricted to the intestine, since recent evidence indicates that oral Bifidobacterium strains (*B. adolescentis*) reduce vitamin K concentration, and may thus compete with *Porphyromonas gingivalis* in the oral cavity [83].

The application of metabolic profiling methods to animal models has suggested another indirect way in which probiotic bacteria might impact on the microbiota, namely, by production of a significantly different microenvironment due to a diverse range of metabolic pathway outcomes. In a recent study using germ-free mice colonized by human baby microbiota and exposed to two lactobacillus strains, Nicholson and colleagues observed microbiome modification, measured by selected culture regimes [84]. This was accompanied by changes in cecal concentrations of short-chain fatty acids, and marked changes in fecal levels of diverse metabolites including choline, acetate, ethanol, a range of putative N-acetylated metabolites (NAMs), unconjugated bile acids (BAs), and tauro-conjugated bile acids. While a natural focus of these studies is the effect of these metabolites

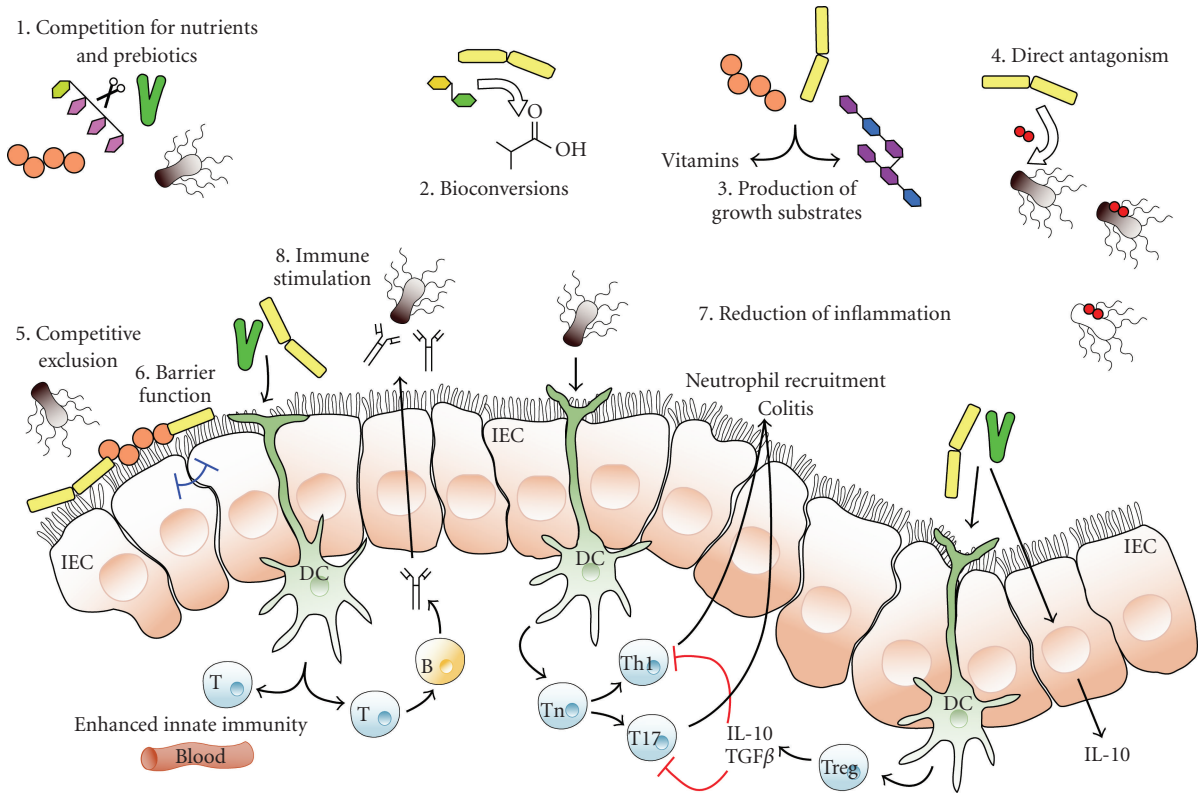

Figure 1: Schematic diagram illustrating potential or known mechanisms whereby probiotic bacteria might impact on the microbiota. These mechanisms include (1) competition for dietary ingredients as growth substrates, (2) bioconversion of, for example, sugars into fermentation products with inhibitory properties, (3) production of growth substrates, for example, EPS or vitamins, for other bacteria, (4) direct antagonism by bacteriocins, (5) competitive exclusion for binding sites, (6) improved barrier function, (7) reduction of inflammation, thus altering intestinal properties for colonization and persistence within, and (8) stimulation of innate immune response (by unknown mechanisms). IEC: epithelial cells, DC: dendritic cells, T:T-cells. For further details, see main text.

upon the host [85], it is likely that such gross changes in metabolic profile also impact upon intestinal microbiota composition. As noted in Table 1, some probiotic bacteria also produce vitamins [27], enhanced availability of which may modulate the microbiota. In addition, exopolysaccharide produced by probiotics including lactic acid bacteria [86] could act as a growth substrate for selected components of the microbiota (see Figure 1).

Probiotic bacteria probably also impact on the general microbiota by direct antagonism. It has been shown in several recent studies that they can modulate numbers of single model organisms in experimental systems. For example, probiotic *L. salivarius* strains inhibit the growth of *H. pylori in vitro* in a strain-dependent manner [17], by mechanisms involving lactic acid secretion, and another as yet uncharacterized mechanism (K. A. Ryan and P. W. O'Toole, unpublished). Intestinal *L. salivarius* strains are distinguished by production of a broad-spectrum bacteriocin Abp118 [87], but this is not likely to contribute to antagonism to Gram-negative bacteria like *H. pylori*. However, production of this bacteriocin Abp118 was identified as the mechanism whereby *L. salivarius* UCC118 eliminated *Listeria monocytogenes* infection in a murine model, providing the first definitive mechanism for anti-infective activity of a probiotic bacterium in vivo [16]. Interestingly, both the wild-type strain UCC118 and a bacteriocin-negative derivative were equally able to suppress *Salmonella* Typhimurium infection in the mouse model, suggesting that broader antimicrobial effects on the Gram-negative components of the microbiota may occur. From an opposite perspective, production of a bacteriocin-like substance by vaginal enterococci has been linked to reduction in levels of commensal lactobacilli that is linked to vaginosis [88]. Natural competition between commensals and opportunistic pathogens may therefore be mediated by mechanisms such as bacteriocin production, that can be exploited for using probiotics to modulate the microbiota. Competitive exclusion (see Figure 1), whereby adherent probiotic species occlude access of members of the microbiota to the epithelium [89, 90], represents another way of modulating the microbiota, although strong evidence for this occurring *in vivo* is lacking.

The most subtle effects wrought by probiotics on the microbiota are potentially those that operate by indirect mechanisms involving the host. Improvement of the intestinal epithelium barrier function [91] might theoretically, for example, impact on efficiency of invasion of pathogens, severity of subclinical tissue damage, and release rates of host-derived micronutrients (see Figure 1), that could

translate into impacts on the microbiota. In an analogous manner, pathological changes in intestinal epithelium might also favor growth of certain members of the microbiota, if inflamed or damaged epithelial cells differentially affect the microbiota. It is well established that some probiotics can suppress inflammation by inhibiting proinflammatory cytokine production [92–94], and although the molecular basis for this is not currently understood for probiotics, mechanisms and molecules have recently been identified in commensals and pathogens [14, 95]. Reduction in gut inflammation by probiotics could plausibly alter the gut environment sufficiently to impact on the microbiota. Furthermore, some probiotic bacteria have been reported to stimulate the innate immune system both in animal models and in elderly subjects [96, 97], by an unknown mechanism. Administration of probiotic bacteria could thus bolster innate immune activity against transient pathogens, or non-commensal elements in the microbiota, leading to subtle changes in long-term overall composition. However, more studies are required to substantiate the mechanisms in the probiotic-host interactions, and to investigate if they do in fact impact on the microbiota.

6. KNOWLEDGE GAPS AND CONCLUDING REMARKS

There has been a rapid recent accumulation of sequence-based information on the composition of the gut microbiota. However, for pragmatic reasons of sample collection facility, this is largely based on fecal analysis, and the microbiota of the colon and small intestine will be different from feces. Studies of the small intestine are particularly warranted because probiotics are proportionally more numerous there, and may exert significant biological activity at this site.

There is adequate information in the literature to support the hypothesis that administration of probiotic cultures in high doses to human subjects will impact on the intestinal microbiota. A comprehensive intervention study, supporting this hypothesis by deep compositional and functional metagenomics approaches, and supplemented by metabolomics, is not currently available (June 2008). In this hypothetical study, mechanisms whereby changes in the microbiota that were achieved could be inferred to a degree by global transcriptional analysis, but definitive linkages between bacterial gene products and effects upon the microbiota could be impossible to establish because of the regulatory issues surrounding human trials with genetically modified organisms. As noted above, proof of principle may be established in animal models, but ultimately these studies must be validated in human subjects. There remains the intriguing question of the role, if any, of the relatively small numbers of potentially probiotic organisms as part of the microbiota in ostensibly healthy individuals. Do these organisms contribute to maintenance of health—or avoidance of disease? Is the level of candidate probiotic organisms in the microbiota critical, and does its importance vary with age? As noted above, there is reasonable evidence that changes in the microbiota accompany disease states like IBD and IBS, conditions whose prevalence increases with aging. There are attractive hygiene-related hypotheses suggesting that depletion of probiotic commensal microbiota in early life may be responsible for the dramatic rise in diseases involving immune dysregulation [98]. The challenge now is to rigorously tackle the interplay of diet, microbiota, and host factors in tractable experiments that will elucidate the key elements in determining outcomes of this interplay, and allow its manipulation.

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