Old Herborn University Seminar Monograph 25:
Bacterial species as partners and pathogens.
Editors: Peter J. Heidt, Tore Midtvedt, Volker Rusch, and James Versalovic.
Old Herborn University Foundation, Herborn-Dill, Germany: 79-90 (2012).

THE MICROBIOTA IN THE DEVELOPMENT OF COLITIS DUE TO *CLOSTRIDIUM DIFFICILE* INFECTION

VINCENT BENSAN YOUNG

Department of Internal Medicine/Infectious Diseases Division,
Department of Microbiology and Immunology,
University of Michigan Medical School, Ann Arbor, MI, USA

SUMMARY

One of the unexpected side effects of antibiotic treatment is the development of colitis. Ranging in severity from nuisance diarrhoea to fulminant pseudomembranous colitis leading to toxic megacolon and perforation, antibiotic-associated colitis is thought to arise from changes in the community structure of the indigenous gut microbiota. The most severe cases of antibiotic-associated colitis are generally due to infection with the toxin-producing bacterium *Clostridium difficile*. Owing to advances in the culture-independent analysis of complex microbial communities that were developed by researchers studying environmental bacterial consortia, we are now in the position to understand how antibiotics alter the indigenous gut microbiota and how these changes lead to susceptibility to *C. difficile* infection. In this review, we will summarize the early work investigating the interaction between *C. difficile* and the gut microbiota and highlight more recent studies using molecular microbial ecology techniques to study *C. difficile* infection humans and animal models of disease.

INTRODUCTION

The development of antibiotics can arguably be regarded as one of the major medical advances of the 20th century. However, as history has shown time and time again technological advances can lead to unintended and unforeseeable consequences. With respect to antibiotics, unintentional alteration of the indigenous microbiota of the host through their broad administration has had significant health effects both acute and chronic. In this review we will discuss one of the earliest observed side effects of antibiotic administration, the development of diarrhoea and colitis.

The development of antibiotics can be viewed as an arms race between researchers in the pharmaceutical industry who are developing novel drugs, often with broader and broader spectrum of activity, and the microorganisms that develop ever-expanding mechanisms of resistance. As the spectrum of activity of the drugs grew in an attempt to circumvent resistance and to allow more effective empiric treatment of life-threatening infections, novel side effects were noted with increasing frequency among patients receiving these treatments. In particular, treatment with the broad-spectrum antibi-

otic clindamycin was associated with a clinical condition that became known as "clindamycin colitis" (*Tedesco* et al., 1974a,b). Patients receiving clindamycin would develop diarrhoea, which in milder cases was simply regarded as a "nuisance" but a proportion of patients would have progressive disease with severe abdominal pain, systemic signs and symptoms and in a minority of cases would progress to toxic megacolon, intestinal perforation and death.

FULFILLING KOCH'S POSTULATES FOR *CLOSTRIDIUM DIFFICILE*

During the 1970s several groups sought to determine the cause of "clindamycin colitis". *Staphylococcus aureus* was one of the first organisms that was implicated as a potential infectious agent causing antibiotic-associated colitis (*Khan* and *Hall*, 1966). Part of this assertion stemmed from the observation that the administration of vancomycin would be an effective treatment for clindamycin colitis. However, the work of two major groups helped fulfil Koch's postulates implicating *Clostridium difficile* as the causative agent of antibiotic associated colitis. These two groups, one led by John Bartlett at Harvard and the other led by Robert Fekety at the University of Michigan, utilized a hamster model of clindamycin-associated colitis (*Bartlett* et al., 1977; *Lusk* et al., 1978). These investigators treated hamsters with clindamycin and subsequently experimentally challenged them with toxin-producing *C. difficile*. This would result in the rapid development of colitis and the subsequent death of the animals thus fulfilling Koch's postulates. These initial animal experiments led to a number of important follow-up studies including the identification of the two major toxins produced by *C. difficile*, establishment of vancomycin and metronidazole as relatively effective treatments for *C. difficile* infection (CDI), the development of diagnostic tests based on the detection of *C. difficile* toxins and the observation that multiple antibiotics could result in subsequent CDI. Thus, in the early 1980s it was felt that much of the major work on *C. difficile* had been completed and much of the attention of infectious disease practitioners and researchers was diverted to other areas, most notably the emerging epidemic of HIV. A number of groups continued to investigate the molecular mechanisms underlying *C. difficile* pathogenesis, most often focused on the biology of the *C. difficile* toxins (*Voth* and *Ballard*, 2005). Renewed interest in this infection came with the recognition of the emergence of a second epidemic of CDI in the early 21st century. This second wave of illness was associated with a previously unrecognized hyperendemic strain of *C. difficile* (denoted as NAP1/BI/027 on the basis of PFGE, REA typing and ribotyping) and increasing severity of disease (*McDonald* et al., 2005; *Pepin* et al., 2004). Simultaneous with the recognition of this new epidemic of CDI, the growing application of research approaches from the field of environmental microbial ecology to host-associated microbial communities as allowed a greater understanding of the pathogenesis of *C. difficile* infection related to changes in the indigenous microbiota secondary to antibiotic administration. We will now review some of the concepts related to microbial ecology and how the indigenous microbiota can mediate "colonization resistance" against pathogenic microbes.

THE INDIGENOUS GUT MICROBIOTA

The indigenous human microbiota includes at least ten thousand different kinds of microbes with a total genetic complexity (the microbiome) that exceeds twenty billion base pairs and codes for more than forty million genes (*Dethlefsen* et al., 2007). Differences in nutrition, host physiology, immunity, state of health and other ecological stressors lead to significantly different microbial population structures and their collective metagenome within and between human hosts. The goal of the Human Microbiome Project is to understand how these complex, dynamic consortia shape human health and well-being (*Turnbaugh* et al., 2007).

The complex community of microbes that inhabits the mammalian gut represents an assemblage of organisms that exists in a balanced symbiosis with its host (*Ley* et al., 2006). The metabolism of the gut microbiota participates in the catabolism of ingested nutrients and also produces a wide variety of ligands and antigens that can interface with the host metabolism and immune system. Therefore, alterations in the community structure of this microbial community can have implications on the homeostasis of the host. Changes in the gut microbiome correlate with obesity, inflammatory bowel disease and antibiotic-associated diarrhoea. These pathologic conditions likely arise via changes in the metabolic activity of the altered microbial community or alterations in the interaction of the microbiome with the host immune system.

THE GUT MICROBIOTA AND COLONIZATION RESISTANCE

The term colonization resistance was coined to refer to the ability of a previously established gut microbial community to resist invasion by an additional microbe (*Freter*, 1962; *Hentges* and *Freter*, 1962; *Vollaard* and *Clasener*, 1994). Although this initially applied to pathogenic microbes, the concept was derived from concepts of community robustness applied to "classical" ecologic systems (for example grasslands and lakes) and thus could be applied to any invading microbe. Current dogma holds that the normal indigenous microbiota is not permissive for colonization of *C. difficile* (*Wilson*, 1993). In the minority of cases where normal individuals are colonized by *C. difficile* without overt clinical disease, it is further hypothesized that the normal indigenous microbiota can at least limit the production of toxin, perhaps by directly interfering with toxin production or limiting the population size of *C. difficile* and preventing significant amounts of toxin from accumulating in the gut. According to this model, disruption of the indigenous microbiota by antibiotics leads to a loss of colonization resistance, making the gut vulnerable to colonization by exogenous *C. difficile* spores or, in previously colonized patients, expansion and toxin production. As noted above, treatment of Syrian hamsters with antibiotics induces a lethal, haemorrhagic colitis (*Bartlett* et al., 1978, 2004). When initially described, this was found to be due to expansion of indigenous *C. difficile* and an increase in toxin production. Wilson and colleagues provided evidence for the ability of the normal gut microbiota to inhibit *C. difficile* by demonstrating that administration of normal caecal homogenates would decrease the num-

ber of viable *C. difficile* and prevent colitis in antibiotic-challenged hamsters (*Wilson* et al., 1981). Subsequent studies in a variety of model systems started to precisely define which members of the indigenous microbiota played a role in mediating colonization resistance. However, the complexity of the gut microbial community and the limitations in the culture-dependent methods that were utilized at the time prevented the performance of more than descriptive studies. The recent development of culture-independent methods of following complex microbial communities and the advent of genomic technologies allows current investigators to revisit hypothesis-driven studies of colonization resistance.

Initial studies of microbial diversity were dependent on the ability to isolate and culture organisms on artificial media. The inability to culture a majority of the microbes present in complex environmental communities has lead to the development of a variety of non culture-based methods for assessing microbial diversity. Over thirty years ago, Woese pioneered the use of rRNA sequence comparisons for charting the evolutionary history of microbes (*Sogin* et al., 1971; *Woese* and *Fox*, 1977; *Woese* et al., 1974). With the introduction of improved sequencing technology, Pace developed culture-independent, molecular tools for assessing the diversity and ecology of microorganisms (*Pace*, 1997; *Pace* et al., 1985). The number of microbial phyla increased from Woese's description of a dozen bacterial lineages to more than 100 major phyla, most of which do not include cultured representatives (*Ley* et al., 2006). Sequence analysis of PCR amplicons for rRNA genes is now the "gold standard" for assessing species richness in microbial communities. Databases contain more than 500,000 rRNA sequences that correspond to phylotypes from diverse microbes*.

Based on surveys using rRNA gene sequences as proxies for the presence of a microbe in a sample, microbial diversity is at least 100-1000 times greater than estimates based upon cultivation-dependent surveys (*Pace*, 1997). Yet these "first generation" molecular assays, involving the amplification, cloning and sequencing16S rRNA-encoding genes, have only captured a fraction of microbial diversity and they rarely provide estimates of relative abundance for differ- ent kinds of microbes or operational taxonomic units (OTUs) (*Pedros-Alio*, 2006, 2007). The occurrence and distribution of the low-abundance taxa remain under-sampled and uncharted (*Pedros-Alio*, 2007). The most extensive molecular surveys of intestinal microbial flora report 13,000-18,000 rRNA gene sequences from humans (*Eckburg* et al., 2005; *Ley* et al., 2006), with one study noting great differences between microbial communities of mucosal surfaces and faeces. Several smaller surveys of gut flora describe correlations with disease states (*Bibiloni* et al., 2006; *Eckburg* and *Relman*, 2007; *Hopkins* et al., 2001; *Zoetendal* et al., 2002), the establishment of gut flora (*Favier* et al., 2003; *Magne* et al., 2006), and effects of antibiotics (*Shoemaker* et al., 2001), etc. However, the limited sampling effort for these investigations is not sufficient to

*http://bioinformatics.psb.ugent.be/webtools/rRNA/
http://www.arb-silva.de
http://ncbi.nlm.nih.gov/
http://greengenes.lbl.gov/cgi-bin/nph-index.cgi)

comprehensively describe the diversity of the human microbiome, and none have addressed the influence of low abundance microbial populations on human health.

Emerging population structure studies of microbial communities from marine environments (*Huber* et al., 2007), soils (*Ashby* et al., 2007; *Roesch* et al., 2007), and the human gut (*Eckburg* et al., 2005; *Ley* et al., 2006) reveal complex diversity patterns where a few dominant phylotypes mask the presence of many thousands of different kinds of lower abundance microbes, many of which represent completely novel OTUs. These studies suggest that even the largest of published metagenomic investigations will not represent the full extent of microbial diversity unless they incorporate normalization procedures to insure the representation of all abundance classes. Implicit in this strategy is the requirement to determine the full complexity of human microbiota or to reduce the population complexity through new cultivation strategies. The descriptions of community complexity must include estimates of the number of different kinds of microbial taxa (richness) and their relative abundance (evenness) for abundant, moderately abundant and rare microbial populations including members of the "rare biosphere". In response to the expense and limited throughput of traditional non culture-based surveys of 16S rRNA-encoding gene diversity, "next generation" technologies (detailed below) have been developed that leverage the power of the newest high-throughput DNA sequencing technologies to provide insight into the rare biosphere. Traditional clone libraries and these next-generation technologies will be employed synergistically in the current proposal to provide a much more comprehensive view of the diversity of the gut microbiota.

MICROBIAL ECOLOGY OF THE GASTROINTESTINAL TRACT UNDER THE INFLUENCE OF ANTIBIOTICS

Human Studies

Although the focus of this discussion is on *C. difficile* , it should be noted that infection with *C. difficile* is estimated to be responsible for only 25% of all cases of antibiotic-associated diarrhoea, although it is generally the cause of the most severe forms of the disease. In the remainder of cases alteration of the normal indigenous microbiota is felt to interfere with the digestion of complex carbohydrates resulting in an osmotic diarrhoea. In one of our initial studies (*Young* and *Schmidt*, 2004) to use culture-independent analysis to profile the gut microbiota we followed changes in the faecal bacterial community of an individual who developed diarrhoea on amoxicillin/clavulanic acid. DNA was purified from faecal samples and the diversity of the bacterial microbiota determined by amplification using broad-range PCR primers that target conserved regions of the 16S rRNA-encoding gene. Our analysis demonstrated that antibiotic administration resulted in dramatic shifts in the composition of the faecal microbiota. Of note there was a marked decrease in members of the family Clostridiaceae. These organisms are known to ferment complex carbohydrates to short chain fatty acids including butyrate, which is the preferred energy source for colonic enterocytes (*Topping* and *Clifton*, 2001; *Wong* et al., 2006). Interestingly, two weeks after the discontinuation of antibiotics the community structure returned largely to baseline.

In a subsequent study we profiled the bacterial microbiota from the faeces of patients diagnosed with antibiotic-associated diarrhoea due to *C. difficile* (*Chang* et al., 2008). In particular, we compared patients who presented with an initial episode of *C. difficile* diarrhoea to patients with recurrent disease. We noted that whereas patients with an initial episode of *C. difficile*-associated diarrhoea had a faecal microbiota with similar diversity to matched controls, the microbiota from patients with recurrent disease exhibited significantly decreased the diversity. These findings provided support for the hypothesis that recurrent *C. difficile*-associated diarrhoea is in at least partly due to persistent abnormalities in the gut microbiota that lead to diminished colonization resistance. Furthermore these findings suggest that the use of faecal transplants for recurrent *C. difficile* infection (*Aas* et al., 2003; *Tvede* and *Rask-Madsen*, 1989) is successful due to restoration of the normal diversity of the gut microbiota and restoration of colonization resistance. A recent study has confirmed this supposition by demonstrating that faecal transplants do have lasting effects on the gut microbiota of the recipient (*Khoruts* et al., 2010).

Other groups have studied the effects of antibiotic administration on the indigenous gut microbiota of healthy volunteers. In general, antibiotic administration has a profound affect on the community structure of the gut microbiota over the short term. There is variability as to the long-term effects of such antibiotic administration. For example, in our initial study we noted that two weeks after the administration of amoxicillin/clavulanate was discontinued, microbial diversity largely returned to the baseline state (*Young* and *Schmidt*, 2004). In another study studying the administration of ciprofloxacin in healthy volunteers, patients had variable recoveries (*Dethlefsen* et al., 2008; *Dethlefsen* and *Relman*, 2011). In some cases, recovery was largely complete following cessation of antibiotics but in other cases patients had long-term changes in their microbiota thus indicating that individual variation in the baseline microbiota and potentially other host factors could influence the response to antibiotic perturbation.

Animal Studies

The above discussion of studies related to antibiotic perturbation in human patients and healthy volunteers suggests that the significant baseline variability of the indigenous microbiota and host genetics has a strong influence on the subsequent behaviour of the microbial community during times of ecologic stress. In an attempt to limit individual-to-individual variation we have turned to inbred murine models to determine if there are reproducible patterns that can be discerned with regards to the response of the gut community to antibiotic perturbation.

In one experiment we utilized a pyrosequencing-based approach that targeted the V6 region of small subunit rRNAs (*Sogin* et al., 2006). The strategy generated over two million V6 tag sequences from bulk DNA extracted from mouse gut tissue. Tag analysis showed that microbial communities of individual co-housed control animals had a remarkably similar structure. The minimal animal-to-animal variation indicates that the gut microbiota in genetically identical animals with the same input microbiota (as all were littermates) follow “assembly rules” that govern the climax structure of the microbial community. This finding is important as the existence of assembly rules implies that variations in commu-

nity structure can provide insight into underlying changes in the gut ecologic environment. For example variables such as host genotype that influences the immunologic state could be reflected in a particular community structure.

Next, we treated inbred mice with two different antibiotic regimens. The first involved a combination of amoxicillin, metronidazole and bismuth and the second was administration of the broad-spectrum cephalosporin antibiotic cefoperazone. Both treatment regimens resulted in significant but distinct shifts in the baseline microbiota (*Antonopoulos* et al., 2009). However, the recovery of the community from these two different stressors was quite distinct. The gut microbiota in animals treated with the combination of amoxicillin, metronidazole and bismuth largely recovered its baseline community structure following the discontinuation of the antibiotics. However, the community in animals treated with cefoperazone remained quite distinct from the baseline even six weeks after the continuation of the drug. The overall biomass of bacteria, which was markedly decreased immediately following the administration of cefoperazone, returned to baseline levels. However, overall diversity of the community remained markedly depressed. These results imply that cefoperazone treatment represents a more severe ecologic stress that AMB administration, a result that could be inferred from the known antimicrobial spectrum of the treatments with cefoperazone having a much broader spectrum. These data indicate that short-term antibiotic administration can have significant effects on the indigenous gut microbiota and these effects may persist long after the discontinuation of the drug.

Murine Models of CDI and the Microbiome

As noted above, Koch's postulates for *C. difficile* and antibiotic-associated colitis were first fulfilled using a hamster model of infection (*Bartlett* et al., 1977). To date this particular animal model has been the most extensively used to study the pathogenesis of *C. difficile*. However, intestinal disease induced in the hamster model is invariably severe, resulting in death within three days after experimental infection. Furthermore, there are limited reagents to study host responses in the setting of hamster infection. For these reasons and others a number of investigators have attempted to develop murine models of *C. difficile* infection. Although mice can be colonized with *C. difficile*, minimal intestinal pathology is encountered which limits the use of this model to study pathogenesis. Germfree mice can be challenged with *C. difficile* and this does result in severe colitis (*Onderdonk* et al., 1980; *Wilson* and *Freter*, 1986) however, this model does not mimic the human situation where a complex indigenous microbiota is present. As such, this system does not let us understand the mechanisms by which the indigenous microbiota mediate colonization resistance, but can provide some insight in to the role of *C. difficile* virulence factors in the disease process.

An important advance in the development of murine models of *C. difficile* infection was recently reported by Chen and colleagues (*Chen* et al., 2008). This group reported that a three day pre-treatment with a cocktail of a five antibiotics (gentamicin, kanamycin, colistin, metronidazole and vancomycin) followed by a two day "washout" period without antibiotics results in a condition where a single dose of

clindamycin followed 24 hours later by experimental infection with *C. difficile* results in the development of colitis. Several aspects of this model make it much more analogous to the situation encountered in human patients compared to the hamster model. Titration of the challenge dose of *C. difficile* can be accomplished to modulate disease severity. In the published study a dose of 10^4 CFU *C. difficile* resulted in a 50% mortality rate. Treatment of *C. difficile*-infected mice with vancomycin, one of the standard treatments for human disease, can prevent mortality although discontinuation of the vancomycin results in disease recurrence. Therefore this model can also be used to model important aspect of human infection with *C. difficile*.

We have recently continued to develop this model of CDI and have used it to study the role of the microbiome in disease pathogenesis (*Reeves* et al., 2011). When we utilized the model as originally described with the cocktail of five antibiotics followed by a single dose of clindamycin prior to experimental infection with *C. difficile* we noted that both treatments were required. If animals received only the five antibiotic cocktail or only a single dose of clindamycin they remained resistant to infection with *C. difficile*, neither being colonized nor developing any clinical or histopathologic evidence of colitis. We compared the microbial ecology of these different antibiotic treatments and found a correlation between loss of members of the phylum Firmicutes and susceptibility to *C. difficile* infection. It is notable that most of the bacteria whose loss was associated with susceptibility to CDI have been postulated to be efficient producers of short chain fatty acids. As previously discussed SCFA is felt to be important for gut homeostasis. We also noted that animals treated with the antibiotic cocktail and clindamycin prior to *C. difficile* infection could follow one of two trajectories. As noted in the original description of the model a certain number of animals would quickly succumb to infection while others appeared to be able to exert some level of control and would not develop clinically severe disease although still were colonized and had histopathologic colitis. When we compared the microbial communities from healthy versus sick animals we noted that animals that appear to be controlling the infection had return of the SCFA-producing Fimicutes while the gut communities in the animals that were clinically ill remained deficient in these organisms.

We also determined if cefoperazone administration, which as noted above could have long-lasting effects on the murine gut microbiota, also led to susceptibility to *C. difficile* infection. We found that cefoperazone alone could lead to a loss of colonization resistance, which was more profound than that induced by the antibiotic cocktail and clindamycin. Animals treated with a course of cefoperazone and then experimentally challenged with *C. difficile* uniformly succumbed to infection within 2 to 3 days. These animals had much higher pathogen levels than animals treated with the antibiotic cocktail clindamycin prior to challenge. Once again, there was a significant loss of the SCFA-producing Firmicutes in cefoperazone treated animals.

CONCLUSIONS

The development of colitis due to *C. difficile* infection following antibiotic

administration represents a disease whose pathogenic mechanism is at the intersection of two schools of thought. Much of the advances we have made in understanding infectious diseases in the past century have been influenced by the theories developed by Robert Koch. We generally think of microbial diseases being the result of infection with a single etiologic agent. Clearly, *C. difficile* infection following antibiotics falls into a disease where a single etiologic agent has been shown to be responsible. However, it is also clear that changes in the complex indigenous microbial community of the gut are also a key feature of the pathogenesis of CDI. We are in the initial stages of understanding what specific changes in the gut community lead to susceptibility to *C. difficile*. Future work defining what functional impact specific changes in microbiota structure have is needed to further understand how alteration of the microbiome leads to CDI. This will also permit us to understand diseases where no single infectious agent can be found and thus pathogenesis is due solely to changes in complex communities. This newer school of thought on the pathogenesis of microbial-associated diseases is only in its infancy and is likely to lead to novel methods for the prevention and treatment of diseases as diverse as obesity, diabetes and inflammatory bowel disease.

LITERATURE

Aas, J., Gessert, C.E., and Bakken, J.S.: Recurrent *Clostridium difficile* colitis: Case series involving 18 patients treated with donor stool administered via a nasogastric tube. Clin. Infect. Dis. 36, 580-585 (2003).

Antonopoulos, D.A., Huse, S.M., Morrison, H.G., Schmidt, T.M., Sogin, M.L., and Young, V.B.: Reproducible community dynamics of the gastrointestinal microbiota following antibiotic perturbation. Infect. Immun. 77, 2367-2375 (2009).

Ashby, M.N., Rine, J., Mongodin, E.F., Nelson, K.E., and Dimster-Denk, D.: Serial analysis of rRNA genes and the unexpected dominance of rare members of microbial communities. Appl. Environ. Microbiol. 73, 4532-4542 (2007).

Bartlett, J.G., Chang, T.W., Gurwith, M., Gorbach, S.L., and Onderdonk, A.B.: Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. N. Engl. J. Med. 298, 531-534 (1978).

Bartlett, J.G., Onderdonk, A.B., Cisneros, R.L., and Kasper, D.L.: Clindamycin-associated colitis due to a toxin-producing species of *Clostridium* in hamsters. J. Infect. Dis. 136, 701-705 (1977).

Bartlett, J.G., Onderdonk, A.B., Cisneros, R.L., and Kasper, D.L.: Comment on J. Infect. Dis. 1977, 136: 701-705; J. Infect. Dis. 190, 202-209 (2004).

Bibiloni, R., Mangold, M., Madsen, K.L., Fedorak, R.N., and Tannock, G.W.: The bacteriology of biopsies differs between newly diagnosed, untreated, Crohn's disease and ulcerative colitis patients. J. Med. Microbiol. 55, 1141-1149 (2006).

Chang, J.Y., Antonopoulos, D.A., Kalra, A., Tonelli, A., Khalife, W.T., Schmidt, T.M., and Young, V.B.: Decreased diversity of the fecal microbiome in recurrent *Clostridium difficile*-associated diarrhea. J. Infect. Dis. 197, 435-438 (2008).

Chen, X., Katchar, K., Goldsmith, J.D., Nanthakumar, N., Cheknis, A., Gerding, D.N., and Kelly, C.P.: A mouse model of *Clostridium difficile*-associated disease. Gastroenterology 135, 1984-1992 (2008).

Dethlefsen, L., Huse, S., Sogin, M.L., and Relman, D.A.: The pervasive effects of an antibiotic on the human gut microbiota, as

revealed by deep 16S rRNA sequencing. PLoS Biol. 6, e280 (2008)..

Dethlefsen, L., McFall-Ngai, M., and Relman, D.A.: An ecological and evolutionary perspective on human-microbe mutualism and disease. Nature 449, 811-818 (2007).

Dethlefsen, L. and Relman, D.A.: Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc. Natl. Acad. Sci. USA 108, Suppl. 1, 4554-4561 (2011).

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., and Relman, D.A.: Diversity of the human intestinal microbial flora. Science 308, 1635-1638 (2005).

Eckburg, P.B. and Relman, D.A.: The role of microbes in Crohn's disease. Clin. Infect. Dis. 44, 256-262 (2007).

Favier, C.F., de Vos, W.M., and Akkermans, A.D.: Development of bacterial and bifidobacterial communities in feces of newborn babies. Anaerobe 9, 219-229 (2003).

Freter, R.: In vivo and in vitro antagonism of intestinal bacteria against *Shigella flexneri*. II. The inhibitory mechanism. J. Infect. Dis. 110, 38-46 (1962)..

Hentges, D.J. and Freter, R.: In vivo and in vitro antagonism of intestinal bacteria against *Shigella flexneri*. I. Correlation between various tests. J. Infect. Dis. 110, 30-37 (1962).

Hopkins, M.J., Sharp, R., and Macfarlane, G.T.: Age and disease related changes in intestinal bacterial populations assessed by cell culture, 16S rRNA abundance, and community cellular fatty acid profiles. Gut 48, 198-205 (2001).

Huber, J.A., Mark Welch, D.B., Morrison, H.G., Huse, S.M., Neal, P.R., Butterfield, D.A., and Sogin, M.L.: Microbial population structures in the deep marine biosphere. Science 318, 97-100 (2007).

Khan, M.Y. and Hall, W.H.: Staphylococcal enterocolitis--treatment with oral vancomycin. Ann. Intern. Med. 65, 1-8 (1966).

Khoruts, A., Dicksved, J., Jansson, J.K., and Sadowsky, M.J.: Changes in the composition of the human fecal microbiome after bacteriotherapy for recurrent *Clostridium difficile*-associated diarrhea. J. Clin. Gastroenterol. 44, 354-360 (2010).

Ley, R.E., Harris, J.K., Wilcox, J., Spear, J.R., Miller, S.R., Bebout, B.M., Maresca, J.A., Bryant, D.A., Sogin, M.L., and Pace, N.R.: Unexpected diversity and complexity of the Guerrero Negro hypersaline microbial mat. Appl. Environ. Microbiol. 72, 3685-3695 (2006a).

Ley, R.E., Peterson, D.A., and Gordon, J.I.: Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124, 837-848 (2006b).

Ley, R.E., Turnbaugh, P.J., Klein, S., and Gordon, J.I.: Microbial ecology: Human gut microbes associated with obesity. Nature 444, 1022-1023 (2006c).

Lusk, R.H., Fekety, R., Silva, J., Browne, R.A., Ringler, D.H., and Abrams, G.D.: Clindamycin-induced enterocolitis in hamsters. J. Infect. Dis. 137, 464-475 (1978).

Magne, F., Abely, M., Boyer, F., Morville, P., Pochart, P., and Suau, A.: Low species diversity and high interindividual variability in faeces of preterm infants as revealed by sequences of 16S rRNA genes and PCR-temporal temperature gradient gel electrophoresis profiles. FEMS Microbiol. Ecol. 57, 128-138 (2006).

McDonald, L.C., Killgore, G.E., Thompson, A., Owens, R.C., Jr., Kazakova, S.V., Sambol, S.P., Johnson, S., and Gerding, D.N.: An epidemic, toxin gene-variant strain of *Clostridium difficile*. N. Engl. J. Med. 353, 2433-2441 (2005).

Onderdonk, A.B., Cisneros, R.L., and Bartlett, J.G.: *Clostridium difficile* in gnotobiotic mice. Infect. Immun. 28, 277-282 (1980).

Pace, N.R.: A molecular view of microbial diversity and the biosphere. Science 276, 734-740 (1997).

Pace, N.R., Stahl, D.A., Lane, D.J., and Olsen, G.J.: Analyzing natural microbial populations by rRNA sequences. ASM News 51, 4-12 (1985).

Pedros-Alio, C.: Marine microbial diversity: Can it be determined? Trends Microbiol. 14, 257-263 (2006).

Pedros-Alio, C.: Ecology. Dipping into the rare biosphere. Science 315, 192-193 (2007).

Pepin, J., Valiquette, L., Alary, M.E., Villemure, P., Pelletier, A., Forget, K., Pepin, K., and Chouinard, D.: *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: A changing pattern of disease severity. C.M.A.J. 171, 466-472 (2004).

Reeves, A.E., Theriot, C.M., Bergin, I.L., Huffnagle, G.B., Schloss, P.D., and Young, V.B.: The interplay between microbiome dynamics and pathogen dynamics in a murine model of Clostridium difficile Infection. Gut Microbes 2, 145-158 (2011).

Roesch, L.F., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K., Kent, A.D., Daroub, S.H., Camargo, F.A., Farmerie, W.G., and Triplett, E.W.: Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J. 1, 283-290 (2007).

Shoemaker, N.B., Vlamakis, H., Hayes, K., and Salyers, A.A.: Evidence for extensive resistance gene transfer among *Bacteroides* spp. and among *Bacteroides* and other genera in the human colon. Appl. Environ. Microbiol. 67, 561-568 (2001).

Sogin, M.L., Morrison, H.G., Huber, J.A., Welch, D.M., Huse, S.M., Neal, P.R., Arrieta, J.M., and Herndl, G.J.: Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc. Natl. Acad. Sci. USA 103, 12115-12120 (2006).

Sogin, S.J., Sogin, M.L., and Woese, C.R.: Phylogenetic measurement in procaryotes by primary structural characterization. J. Mol .Evol. 1, 173-184 (1971).

Tedesco, F.J., Barton, R.W., and Alpers, D.H.: Clindamycin-associated colitis. A prospective study. Ann. Intern. Med. 81, 429-433 (1974a).

Tedesco, F.J., Stanley, R.J., and Alpers, D.H.: Diagnostic features of clindamycin-associated pseudomembranous colitis. N. Engl. J. Med. 290, 841-843 (1974b).

Topping, D.L. and Clifton, P.M.: Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol. Rev. 81, 1031-1064 (2001).

Turnbaugh, P.J., Ley, R.E., Hamady, M., Fraser-Liggett, C.M., Knight, R., and Gordon, J.I.: The human microbiome project. Nature 449, 804-810 (2007).

Tvede, M. and Rask-Madsen, J.: Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients. Lancet 1, 1156-1160 (1989).

Vollaard, E.J. and Clasener, H.A.: Colonization resistance. Antimicrob. Agents Chemother. 38, 409-414 (1994).

Voth, D.E. and Ballard, J.D.: *Clostridium difficile* Toxins: Mechanism of Action and Role in Disease. Clin. Microbiol. Rev. 18, 247-263 (2005).

Wilson, K.H.: The microecology of *Clostridium difficile*. Clin. Infect. Dis. 16, Suppl. 4, S214-S218 (1993).

Wilson, K.H. and Freter, R.: Interaction of *Clostridium difficile* and *Escherichia coli* with microfloras in continuous-flow cultures and gnotobiotic mice. Infect. Immun. 54, 354-358 (1986).

Wilson, K.H., Silva, J., and Fekety, F.R.: Suppression of *Clostridium difficile* by normal hamster cecal flora and prevention of antibiotic-associated cecitis. Infect. Immun. 34, 626-628 (1981).

Woese, C.R. and Fox, G.E.: Phylogenetic structure of the prokaryotic domain: The primary kingdoms. Proc. Natl. Acad. Sci. USA 74, 5088-5090 (1977).

Woese, C.R., Sogin, M.L., and Sutton, L.A.: Procaryote phylogeny. I. Concerning the relatedness of *Aerobacter aerogenes* to *Escherichia coli*. J. Mol. Evol. 3, 293-299 (1974).

Wong, J.M., de Souza, R., Kendall, C.W., Emam, A., and Jenkins, D.J.: Colonic health: Fermentation and short chain fatty acids. J. Clin. Gastroenterol. 40, 235-243 (2006).

Young, V.B. and Schmidt, T.M.: Antibiotic-associated diarrhea accompanied by large-scale alterations in the composition of the

fecal microbiota. J. Clin. Microbiol. 42, 1203-1206 (2004).

Zoetendal, E.G., von Wright, A., Vilpponen-Salmela, T., Ben-Amor, K., Akkermans, A.D., and de Vos, W.M.: Mucosa-associated bacteria in the human gastro-intestinal tract are uniformly distributed along the colon and differ from the community recovered from feces. Appl. Environ. Microbiol. 68, 3401-3407 (2002).