THE PATHOLOGICAL MICROBIOTA

The Microbiota and infectious diarrhea ✯

Le microbiote dans les diarrhées infectieuses

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Summary Understanding the importance of the fecal microbiota has been key in understanding the pathophysiology of some infectious diarrheas. In addition to normal protective measures of bile, gastric acid, and immune response, among others, we now know that the healthy gut flora protects us from some infectious diarrheas. Antibiotic associated diarrhea (AAD) is an excellent example, as antibiotics perturb the normal flora; the resulting diarrhea may be due to changes in short chain fatty acid metabolism. A severe form of AAD is due to Clostridium difficile, a pathogen that can cause severe diarrhea, colitis and even death. Recurrent Clostridium difficile diarrhea is a difficult clinical problem to treat successfully because one recurrence makes further recurrences more likely, probably because antibiotics are still needed to treat and thus the fecal flora remains abnormal. There is no single effective treatment but therapies include pulsed and tapered antibiotics, the probiotic Saccharomyces boulardii as an adjunct to antibiotics, and even fecal flora reconstitution. It is likely that we will learn even more in the future about the beneficial effect of our microbiota.

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Résumé La compréhension de l’importance du microbiote fécal a été un élément clé pour comprendre la physiopathologie de certaines diarrhées infectieuses. Outre le rôle protecteur normal de la bile, de l’acidité gastrique et de la réponse immunitaire, entre autres, nous savons maintenant que la flore intestinale normale nous protège de certaines formes de diarrhées infectieuses. La diarrhée associée aux antibiotiques (DAA) en est un excellent exemple, car les antibiotiques perturbent la flore normale. La diarrhée qui en résulte pourrait être due à des modifications du métabolisme des acides gras à chaîne courte. La diarrhée liée à Clostridium difficile, agent pathogène qui peut induire une diarrhée grave, une colite et même le décès, est une forme particulièrement sévère de DAA. La diarrhée récidivante à Clostridium difficile est un problème clinique difficile à traiter efficacement, car chaque récidive augmente la probabilité d’un nouvel épisode, probablement du fait que la prescription d’antibiotiques reste nécessaire pour la traiter et que la flore intestinale reste de ce fait perturbée. Il n’y a pas d’attitude thérapeuti que efficace unique. Les possibilités thérapeutiques incluent l’utilisation d’antibiotiques

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bénéfiques du microbiote.
Il faut s’attendre à voir nos connaissances s’enrichir encore dans l’avenir sur les effets bénéfiques du microbiote.
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Introduction

Infectious diarrhea is an important global health challenge. The World Health Organization reported that gastrointestinal infections accounted for 1.5 million deaths in 2007. Over 70% of gastrointestinal infections are food borne. There are over 200 organisms that cause diarrhea, some of which have not yet been fully indentified. In the developing world infections can result in malnutrition, recurrent infections, malabsorption, increased susceptibility to other infections, growth retardation in children, resulting in both immediate and delayed morbidity and mortality. Also, in the developing world, infections may trigger irritable bowel syndrome and inflammatory bowel disease.

Protection from enteric infections

Humans are protected from pathogens by the mucosal barrier including mucin, the systemic immune response and the local immune system, e.g. the gut associated lymphoid tissue (GALT), and our fecal microbiota [1, 2]. The mucosa is our major physical barrier to ingested pathogens. The surface epithelial cells and mucin provide a physical barrier. Another mechanism of protection is the secretion of antimicrobial peptides into the lumen. These peptides, called defensins, epithelial cells and mucin provide a physical barrier. Another major physical barrier to ingested pathogens. The surface epithelial cells and mucin provide a physical barrier. Another mechanism of protection is the secretion of antimicrobial peptides into the lumen. These peptides, called defensins, are secreted by Paneth cells. Other protective mechanisms include gastric acid as well as bile and pancreatic enzymes and intestinal motility. Recent research suggests that some genes are implicated in our susceptibility to enteric infections [3].

Gastric acid plays a role in protecting us from ingested pathogens, presumably killing some pathogens we ingest. This may have been more important in the past from an evolutionary point of view. Patients who have had a partial gastrectomy and thus are achlorhydric are more susceptible to enteric infections such as Salmonella. A study of individuals with enteric infections in Bangladesh correlated cholera infection with decreased gastric acidity [4]. Currently, low gastric acid is more often due to medications, and the proton pump inhibitors decrease gastric acid profoundly. Proton pump inhibitors (PPIs) are widely used in the treatment of peptic ulcer disease and gastroesophageal reflux disease, and as a group are among the most widely prescribed drugs in the US. A recent systematic review showed that PPIs predisposed to enteric infections, including Clostridium difficile [5]. Peristalsis is another important mechanism, with mechanical removal of pathogens, and diarrhea itself may help to mechanically clear pathogens. In addition, in animal models of enteric infection, antimotility agents delayed clearing of pathogens such as Shigella [6] and there have been cases of severe toxic colon in individuals with dysentery given antidiarrheals. Diseases that have decreased motility such as scleroderma are associated with bacterial overgrowth, with diarrhea due to bacterial deconjugation of bile salts among other mechanisms. Primary bile acids are converted to secondary bile acids in the colon by bacteria including Clostridium spp and Eubacterium spp; if this conjugation is impaired, more primary bile acids in the colon can result in a secretory diarrhea. Systemic and local immune responses are import defenses against enteric infections. Immunosuppressed individuals are more susceptible to infections, and infections can be more severe, and even fatal. In addition, these patients can develop disease from organisms that are usually not pathogenic, such as cytomegalovirus (CMV). The local immune response is also important. For example recurrent giardiasis is associated with IgA immune deficiency syndrome. There is increasing recognition of the importance of the fecal microbiota in protecting us from infection. The microbiota consist of over 10-100 trillion cells, with over 1000 different strains each in our gut and on our skin. Our microbiota encodes physiologic capabilities that the human genome lacks. While there are over 70 candidate bacterial phyla, there are only 10 in the human gut. Most (80%) cannot be cultured. There are two phyla that predominate: the Firmicutes and the Bacteroidetes account for 90-99% of all bacteria. The microbiota has many important functions. The distal gut microbiota metabolizes plant carbohydrates that we cannot degrade, producing short chain fatty acids, SCFAs, (propionate, acetate and butyrate) as well as other metabolites - carbon dioxide, hydrogen sulfide, formate and others. The short chain fatty acids retrieve 10% of our caloric intake and provide nutrition for our enterocytes, aid fluid absorption in the colon. The flora also deconjugate bile salts, salvage urea and synthesize amino acids, B vitamins and vitamin K, and are important for development of the immune system, as well as angiogenesis. Importantly the microbiota protects us from the germs in that we ingest every day. For decades the study of the fecal flora used quantitative and qualitative stool cultures. A summary of the effect of various antibiotics on the normal flora culled from studies of many different antibiotics can be found in two reviews [7, 8]. Newer technology uses 16S rDNA to evaluate the microbiota diversity and alterations. All bacteria have one or more 16S rDNA genes that encode for 16S ribosomal DNA. Combinations of highly conserved and highly variable sequences in a short part of the bacterial chromosome provide a tool to measure diversity.

Evidence for the importance of the microbiota comes from germ free animals and from animal models where the flora is altered by antibiotics. Germ free mice are more susceptible to infection. For example, guinea pigs are not...
normally susceptible to cholera infection, but when given the antibiotic streptomycin they do become susceptible [9]. Moreover, in a mouse model with \textit{Shigella flexneri} colonization, there were $10^9$ grams of \textit{Shigella} compared to $10^5$ in precolonized mice and $10^7$ in mice precolonized with \textit{Escherichia coli} [10]. The hamster model of clindamycin colitis led to the discovery of \textit{Clostridium difficile} [11]. Colonization resistance is the ability of the flora to protect us from infection. There are many putative mechanisms involved. Some of these are stabilization of the luminal pH, producing inhibitory substances such as toxic metabolites and antimicrobial peptides such as bacteriocins, limiting nutrients to pathogens, blocking attachment sites to pathogens, stimulating mucus secretion and production of signal molecules that act on genes encoding survival [12-14].

Bacterial production of short chain fatty acids is very important. \textit{Bifidobacteria} and \textit{Lactobacilli} are important producers of SCFA. In \textit{in vitro} experiments, SCFAs inhibit growth of pathogens including \textit{Escherichia coli}, \textit{Salmonella}, \textit{Bacillus cereus} and \textit{Clostridium difficile}. Raqib et al. showed that one SCFA, butyrate, reduced the antimicrobial peptide cathelin in LL37 resulting in elimination of \textit{Shigellos spp in vitro} [15]. The microbiota produces inhibitory substances; bacteriocins are antibacterial proteins that have a broad inhibitory spectrum for gram positive and gram negative species. For example, \textit{Lactobacillus reuteri} produce reuterein, a bacteriocin that inhibits \textit{Salmonella}, \textit{Shigella}, \textit{Clostridia} and \textit{Listeria in vitro}. Anaerobic bacteria can produce hydrogen peroxide that causes peroxidation of the lipid membrane which increases bacterial membrane permeability allowing destruction of the bacterial nucleic acids. For example in the vagina, lactobacilli use this mechanism to inhibit \textit{Chlamydia}. Theoretically the microbiota competes with pathogens for nutrients. This is difficult to study. In a continuous flow culture model in mice, the flora competed for glucose and saccharic acid, resulting in decreased levels of \textit{Clostridium difficile} [16]. Diet can affect our microbiota. Dietary concentrations of fat, fiber or protein can alter the numbers of primary or secondary fermenters, with subsequent changes in inhibitory substances such s SCFA. One example of the importance of diet comes from the analysis of the microbiota in obese and non obese individuals. The obese individuals had more \textit{Firmicutes} and fewer \textit{Bacteroidetes} and this ratio changed with a change in diet [17]. Further evidence for the role of diet comes from studies of prebiotics, which are dietary substances that, when ingested, stimulate the growth of beneficial bacteria. For example, ingestion of inulin and oligofructose in humans resulted in increased numbers of \textit{Bifidobacteria} (18). The flora can compete with pathogens for attachment sites (mucin adhesion sites). The intestine is coated with a layer of mucus made up of glycoprotein. This can trap bacteria; adhesion sites for beneficial bacteria are available for the adhesins on the bacteria. The host genome controls the flora by genetic control of adhesion sites. In an animal model, colonization with nonpathogenic bacteria attachment and prevented invasion by enterovirulent bacteria [19]. More exciting research exploring the communal host relationship in the gut showed that the resident flora can send messages to host cells that change the carbohydrate in mucus; this could affect whether pathogenic or nonpathogenic bacteria can adhere [20].

**Pathogenesis of infectious diarrhea**

With all these protective measure and others likely as yet unidentified, how do pathogens succeed in causing illness? This depends on pathogen virulence factors as well as host defense factors. Bacteria have a variety of virulence factors. They can produce enterotoxins (\textit{Shigella}), cytotoxins (\textit{Clostridium difficile}) or both. They can adhere to the mucosa (\textit{Giardia lamblia} and enteroadherent \textit{Escherichia coli}). They can invade the mucosa, causing cell injury, inflammation and ulceration (\textit{Shigella}). These are just a few of the bacterial virulence factors. Viruses and parasites have other methods to cause disease. Pathogens can circumvent the protective mechanisms. Recent research suggests they can even use the host inflammatory response to foster their own growth. This has been shown in animal models using two different pathogens. \textit{Salmonella} enteric serovar typhimurium and murine \textit{Citrobacter rodentium} (which is similar to enteropathogenic \textit{Escherichia coli}) [21, 22]. In experiments, neither pathogen colonized non inflamed gut. When colonized with avirulent strains they were outgrown by the normal microbiota in 4 days. When the gut was inflamed, both in a colitis model and with virulent \textit{Salmonella}, the pathogen became dominant [21], indicating that the inflammatory response allowed the pathogen to dominate. In mice infected with \textit{Citrobacter rodentium}, there was a decrease in the normal microbiota, mainly due to the activation of the host immune response [22].

**Microbiota and infectious diarrhea**

Detailed studies of the microbiota in humans with enteric infections are limited. In one study of children with acute gastroenteritis in India, changes in the flora during the diarrheal phase of the illness were documented with decreases in \textit{Eubacteria} and \textit{Bacteroides}, and normalization by 4 weeks [23].

**Antibiotic associated diarrhea**

Antibiotics alter the microbiota and are a good model for study, proving the importance of the flora. Perhaps the best known is the effect of clindamycin on hamsters. When the antibiotics lincomycin and clindamycin were introduced in the late 1960s and 1970s, some individuals became ill with a severe colitis with pseudomembranes seen on colonoscopy or autopsy. This entity, initially called lincomycin colitis and later clindamycin colitis is best known as pseudomembranous colitis (PMC). Hamsters given clindamycin became ill with cecitis; experiments in this model led to the discovery of the causative organism, \textit{Clostridium difficile}, a gram positive anaerobe, as the cause of PMC in humans. Not only do antibiotics cause diarrhea and \textit{Clostridium difficile} infection, they can also increase the host susceptibility to infection. In addition, mice given antibiotics get sicker with smaller inclusions of pathogens. Mice given streptomycin and vancomycin were more susceptible to \textit{Salmonella} and \textit{Clostridium difficile} infection [24]. Antibiotics promote the growth of antibiotic resistant flora which can exert
selective pressure that alters the flora [25]. Other theories to explain this include changes in the immune response or selective removal of bacteria that are barriers. While the flora usually normalizes by 4 weeks after antibiotics are stopped, there can be longer term changes. The changes in the flora have effects on carbohydrate metabolism. In mice given vancomycin, Yap et al documented changes in carbohydrate fermentation of oligosaccharides and decreased SCFAs in the stool as a result of changes in the microbiota [26].

In humans antibiotics are a known cause of diarrhea, occurring in up to 25% in outpatients and 39% in hospitalized patients, depending on the antibiotic used among other factors. Antibiotics that have a broad spectrum and alter the anaerobic flora more profoundly are more likely to cause diarrhea. Amoxicillin, clindamycin and third generation cephalosporins are most likely to cause antibiotic associated diarrhea (AAD). In healthy volunteers given antibiotics the fecal flora can be analyzed. Volunteers given amoxicillin for 5 days had decreases in the diversity of the microbiota that occurred early, within 2-3 days [27] (Fig. 1). Volunteers given Clindamycin had decreased clonal diversity of the flora compared to controls. Bacteroidetes were the most subject to variability. Flora usually normalized by 4 weeks but could rarely be abnormal up to 2 years [28]. Prospective analysis of stools collected during and post antibiotic therapy from a man who developed AAD documented decreased levels of two clostridial clusters (IV and XIVa) that are usually predominant [29]. In this case, the microbiota normalized after the antibiotics were stopped and the diarrhea resolved.

Probiotics are living organisms that, when ingested, are beneficial to the host. Their use and studies of their efficacy provide additional evidence of the importance of the microbiota as they affect the flora. Probiotics have been shown to prevent AAD, with excellent data for Saccharomyces boulardii and to a lesser extent Lactobacillus GG [30]. Other agents are less effective and less well studied; these include Bifidobacteria, Bacillus clausii, Lactobacillus acidophilus, Enterococcus faecium, Clostridium butyricum and mixtures of probiotics. In recent studies of yogurt, there was a decrease in incidence of AAD in one study [31] but no prevention of AAD in another [32]. Probiotics have been studied as well. While promising in animal models, fructo-oligosaccharide supplementation did not decrease AAD even though it did increase concentrations of Bifidobacteria [33].

**Clostridium difficile infection**

*Clostridium difficile* infection (CDI) is a severe type of AAD caused by the gram positive anaerobe bacterium that produces toxins that cause disease. The spectrum of symptoms can range from mild diarrhea to severe pseudomembranous colitis that can be fatal. While usually a consequence of antibiotic therapy other causes are cancer chemotherapy, as well as cases with no prior antibiotic use. Since 2000 there has been an epidemic associated with a strain Nap 1 BI/027 (ribotype) that has caused epidemics with increased morbidity and mortality. The first documented epidemic was in a Pittsburgh teaching hospital where there was a marked increase in severe and fatal cases of *Clostridium difficile* in 2000. In this epidemic, quinolone use was identified as a major risk factor [34]. Two years later, in 2002, there was a dramatic increase in numbers and severity of CDI cases in

![Figure 1](image-url)  
**Figure 1** Evolution of dominant fecal microbiota during and after antibiotic treatment (similarity indices (%) of TTGE profiles of volunteers from D1 to D60. n, number of subjects tested) (from [27] with permission).
Quebec, where there have been thousands of cases. This has been linked to an epidemic strain, documented in most states in the US, parts of Canada, as well as many countries in Europe and in Japan [35]. The first documented case in Japan was in 2005 in a patient with inflammatory bowel disease. The epidemic strain produces more toxins A and B in vitro which may account for its increased virulence and in addition to clindamycin resistance it has acquired quinolone resistance. Increased use of quinolone antibiotics in the past decade may have selected for this strain. Treatment of CDI with antibiotics, usually metronidazole or vancomycin is necessary to treat the CDI. Although there have been reports of decreased response rates of metronidazole, probably associated with severity of disease initial treatment with metronidazole is recommended. In patients with severe disease, initial treatment with vancomycin is recommended, as well as for those who do not respond to metronidazole within three days. In severe cases colectomy can be life saving.

Well recognized risk factors for CDI are exposure to antibiotics or chemotherapy, hospitalization, older age, immunosuppression and comorbid conditions. *Clostridium difficile* disproportionately affects those 65 or older, with the highest rates and highest mortality in those over 85 years of age. Possible explanations are relative immune suppression or changes in the microbiota. In vitro, polymorphonuclear cells from elderly subjects had less ability to kill *Clostridium difficile* than those from younger subjects [36]. As for the microbiota, studies in older subjects showed no change in total CFU or changes in *Bifidobacteria* or *Lactobacilli* but decreases in *Bacteroides* and increases in *Escherichia coli* and *Enterococci* [37]. Since the epidemic new risk factors have appeared: sporadic cases in young healthy low-risk individuals including a small series in 10 pregnant women with severe disease, more case without prior antibiotic use, patients with inflammatory bowel disease (IBD), cirrhosis and possible use of proton pump inhibitors (PPIs). Several recent papers have documented an increased incidence of CDI in patients with IBD, with worse outcomes and increased mortality compared to either IBD or CDI alone [38]. In two series, rates of CDI dramatically increased, doubling in patients with Cohn’s disease and tripling in the ulcerative colitis patients. More patients went to urgent colectomy, and risk factors were colon disease and immunosuppressive therapy [39, 40]. A recent publication documents increased morbidity in patients with cirrhosis who have CDI, compared to either cirrhosis or *Clostridium difficile* alone. Risk factors were antibiotic use and PPIs [41]. The association of PPI use and CDI has been a subject of debate recently with many studies with conflicting results. Studies have used both inpatient and outpatient data bases, case controlled, but methods vary and some have been done during epidemics which may affect results. Studies using outpatient data bases are more appealing because they remove confounding factors of hospitalization and severe illness. Two large studies had conflicting results; one used the UK GPRD data base and showed an association of PPI use and CDI [42] and the other used an Ontario Canada data base and did not show an association [43]. This may reflect difference in patient populations, or severity of disease since the UK data base study used vancomycin as a surrogate for diagnosis of CDI and this would be used for more severely ill patients. The recent systematic review of acid suppression and risk of enteric infections correlated the two, and includes *Clostridium difficile* as well [5]. Proof of an associate would require a prospective controlled trial which is unlikely to be done. In the meantime judicious use of PPIs is suggested.

Some cases of AAD are not due to *Clostridium difficile*. There is an entity called antibiotic associated hemorrhagic colitis (AAHC), first described in 1978, often associated with penicillin or penicillin-derivative antibiotics. Several cases of AAHC in which *Klebsiella oxytoca* was cultured have been reported. In a series of 22 patients with suspected antibiotic associated colitis, 6 had colitis after penicillin derivatives, and 5 had *Klebsiella oxytoca* cultured from stools [44]. These investigators also gave *Klebsiella* from one of the patients to rats given amoxicillin clavalanate; the rats developed right sided hemorrhagic colitis, thought not the rats without antibiotic pretreatment in whom there was no colonization or colitis. A recent case report shows pseudomembranous colitis in a patient with *Klebsiella oxytoca* colitis [45].

Another potential cause of nosocomial diarrhea is enterotoxin-producing methicillin resistant *Staphylococcus aureus* (MRSA). A prospective study of hospitalized patients who developed AAD but did not have *Clostridium difficile* had stools tested for MRSA. Eleven patients were identified with enterotoxin-producing MRSA in their stool. The toxins in the stool were the same as those from the strain identified from their stools. They had more diarrhea than those with non enterotoxigenic strains of MRSA, and toxin levels correlated with severity of diarrhea [46].

Most patients with CDI will respond to antibiotics. However, 10-20% will develop recurrent symptoms after finishing their antibiotics. This is called recurrent CDI (RCDI) although early literature uses the term relapsing *Clostridium difficile*. After one recurrence, further recurrence rates increase to 40-60%. This requires repeated courses of antibiotics and sets up a vicious cycle of recurrences that is likely due to persistently abnormal flora. This theory is supported by a study of the microbiota in 7 patients with CDI and 3 controls. In those who developed RCDI, there was a significant and consistent decrease in phylogenetic richness of the flora compared to controls and to those with CDI who did not develop recurrences [47] (Fig. 2). Immune response may play a role. In a small series of patients with CDI those who developed recurrence had lower levels of IgM and IgG to toxin A than those who did not recur [48].

Therapy of RCDI can be a challenge since there is no uniformly effective therapy. Therapy also gives a clue to pathophysiology. Repeat antibiotics are necessary but when given in a tapering and pulse fashion recurrences are less frequent [49] suggesting that the flora can normalize on the days of antibiotics (and that perhaps dormant *Clostridium difficile* spores germinate on those days and are later killed by the antibiotics but this is only a theory). Immune approaches have been tried with anecdotal success—inc luding IVIG, bovine whey, supporting an immune role in pathophysiology. The probiotic *Saccharomyces boulardii* has efficacy, supporting a role of the microbiota. In two trials of RCDI, *Saccharomyces boulardii* as an adjunct to antibiotics (metronidazole or vancomycin) decreased recurrences by
Figure 2. Analysis of 16S clone libraries of the fecal microbiota in patients with antibiotic-associated diarrhea due to Clostridium difficile (from [47] with permission).
A- Relative abundance of members of predominant bacterial phyla, in fecal samples
B- Rarefaction analysis of 16S clone libraries from each individual in the study. Phylotypes were based on an operational taxonomic-unit definition of 97% sequence identity.
50% in one trial [50]. In a later trial it was efficacious in a subgroup also given high dose vancomycin, decreasing recurrences to 15.7% compared to 50% in controls [51], but was not efficacious when paired with low dose vancomycin or metronidazole [52]. Lactobacillus GG has been shown effective anecdotally but in 2 controlled trials it was not efficacious [53, 54]. Finally, Lactobacillus plantarum decreased recurrences in a small trial [52]. There is a small controlled study in hospitalized patients using a prebiotic; those given the prebiotic with their antibiotic had fewer recurrences and shorter hospital stays [55].

Fecal bacteriotherapy has received much recent attention. An early report of fecal enemas in seriously ill patients with CDI using donor stool from healthy individuals reported success [56]. In 1983, Schwan et al reported successful treatment of RCDI in one patient given a fecal enema [57]. Tvede et al used fecal bacteriotherapy using a mixture of 10 facultative aerobic and anaerobic bacteria to successfully treat 6 patients with RCDI [58]. Persky and Brandt reported resolution of RCDI in a woman after stool from her healthy spouse was infused into her colon via a colonoscope [59]. Two series using stool from healthy donors delivered by nasogastric tube into persons with RCDI have been reported. The first was by Aas et al, reporting success in 16 of 18 patients studied [60]. A more recent paper reports that 11 of 15 were cured with this approach [61]. This author has had experience in 7 patients with RCDI using donor stool delivered via colonoscopy with success in all, with follow up from 10 months to 5 years. Interestingly, 6 of the 7 were women (Surawicz CM, unpublished data). This experience supports the theory that abnormalities of flora are important in pathogenesis of RCDI.

Conclusion

In summary, the microbiota is important in protecting us from infection. AAD and RCDI are good models to study. Further exploration of the microbiota will result in novel ways to prevent and treat such infections. Future directions in prevention and treatment of enteric infections are clean food and water, early diagnosis, better treatments, such as inexpensive and available oral rehydration solutions, antibiotics when appropriate, vaccines and probiotics and prebiotics.

Conflicts of Interests

The author has received honoraria for speaking from Biocodex Inc.

References


