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Microbial ecosystems therapeutics: a new paradigm in medicine?

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Abstract

Increasing evidence indicates that the complex microbial ecosystem of the human intestine plays a critical role in protecting the host against disease. This review discusses gut dysbiosis (here defined as a state of imbalance in the gut microbial ecosystem, including overgrowth of some organisms and loss of others) as the foundation for several diseases, and the applicability of refined microbial ecosystem replacement therapies as a future treatment modality. Consistent with the concept of a 'core' microbiome encompassing key functions required for normal intestinal homeostasis, 'Microbial Ecosystem Therapeutics' (MET) would entail replacing a dysfunctional, damaged ecosystem with a fully developed and healthy ecosystem of 'native' intestinal bacteria. Its application in treating *Clostridium difficile* infection is discussed and possible applications to other diseases such as ulcerative colitis, obesity, necrotising enterocolitis, and regressive-type autism are reviewed. Unlike conventional probiotic therapies that are generally limited to a single strain or at most a few strains of bacteria 'Microbial Ecosystem Therapeutics' would utilise whole bacterial communities derived directly from the human gastrointestinal tract. By taking into account the intrinsic needs of the entire microbial ecosystem, MET would emphasise the rational design of healthy, resilient and robust microbial communities that could be used to maintain or restore human health. More than simply a new probiotic treatment, this emerging paradigm in medicine may lead to novel strategies in treating and managing a wide variety of human diseases.

Keywords: human gut microbiota, dysbiosis, complex probiotics

1. Introduction

The human gastrointestinal tract contains vast numbers of bacteria collectively called the intestinal microbiota (Ley *et al.*, 2006a). It is becoming more and more evident that the intestinal microbiota plays an important role in health and this may reach beyond its influence on intestinal health. A variety of factors including the combined effects of antimicrobial overuse both in the agricultural and clinical setting have taken a toll upon our microbial diversity (Figure 1) and increasing evidence from a variety of conditions ranging from obesity to antibiotic-associated infectious diarrhoea (including pseudomembranous colitis) indicate that the complex microbial ecosystem of the intestine plays a critical role, either directly or indirectly, in protecting the host against disease. Consistent with the concept of a 'core' microbiome (Turnbaugh *et al.*, 2007, 2009) that encompasses key functions required for normal intestinal homeostasis 'Microbial Ecosystem Therapeutics' (MET) would entail replacing a dysfunctional, damaged ecosystem with a fully developed and healthy ecosystem composed of dozens of strains of 'native' intestinal bacteria. Unlike conventional probiotic therapies that are generally limited to a single strain or at most a few strains of bacteria, MET would utilise whole bacterial communities that have been derived directly from the gastrointestinal tract. More than simply a new probiotic treatment, this emerging paradigm in medicine may lead to novel strategies in

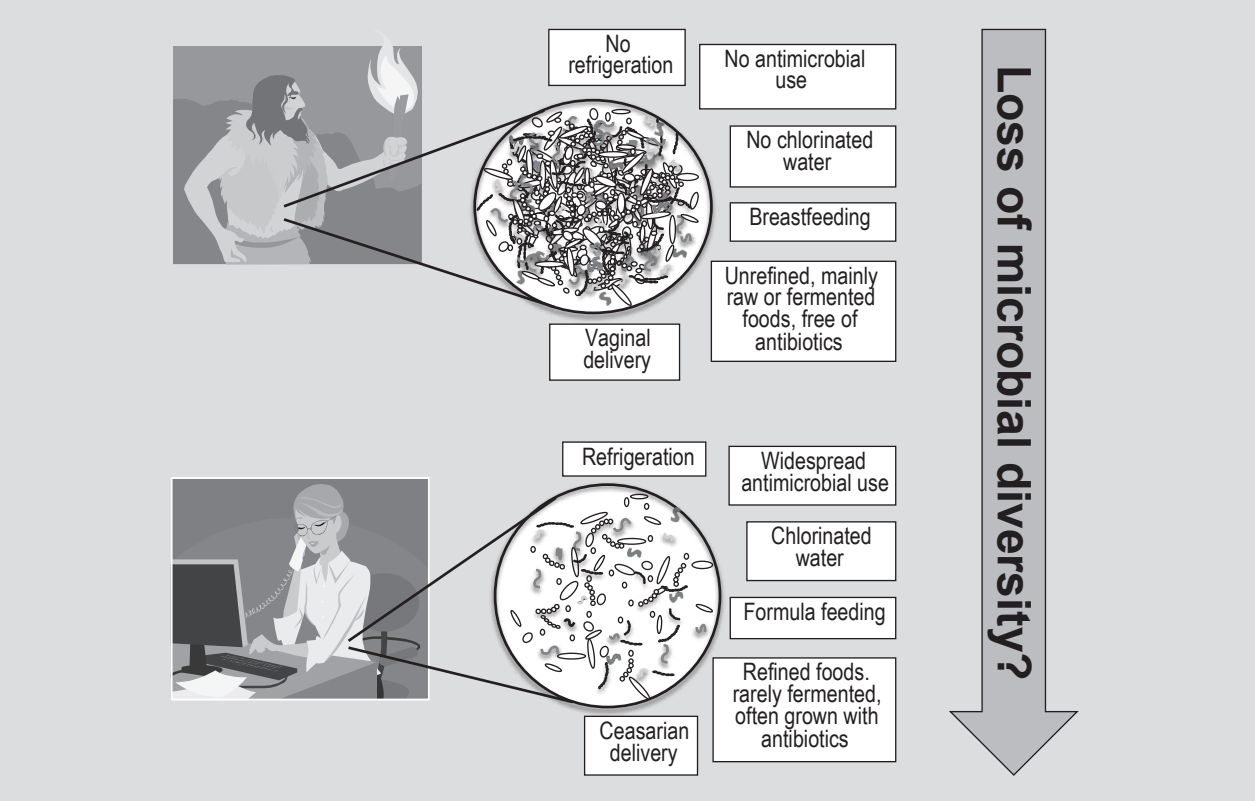

Figure 1. Are diseases associated with gut microbial ecosystem dysbiosis in turn related to loss of microbial diversity? Modern practices that have established a potential influence on microbial diversity are depicted here.

treating and managing a wide variety of human diseases. This review seeks to summarise what is known about gut dysbiosis (defined as a state of imbalance in the gut microbial ecosystem, including overgrowth of some organisms and loss of others), as the foundation for several diseases and to pose important unanswered questions about the applicability of refined microbial ecosystem replacement therapies as a future treatment modality.

2. Faecal transplants to treat *Clostridium difficile* infection: the original Microbial Ecosystem Therapeutics approach

Our commensal intestinal bacteria form a microbial ecosystem that contributes to host defence by priming the dendritic cells of the immune system, by producing bactericidal products that kill other pathogenic bacteria and volatile fatty acids that inhibit the colonisation of pathogenic bacteria, and by competing with pathogens for food and binding sites along the intestinal epithelial cell surface, a phenomenon collectively known as 'colonisation resistance' (Rolfe, 1984; Stecher and Hardt, 2008). This concept is well illustrated by faecal microbial therapy, which has been used to treat recurrent *Clostridium difficile* infection (CDI).

CDI is a bacterial infectious disease of the gastrointestinal tract caused by *C. difficile*, a toxin-producing Gram-positive anaerobic, spore-forming bacillus. When mild, CDI causes abdominal pain and diarrhoea; when severe, CDI causes colitis that can be life-threatening. CDI accounts for 15-25% of antibiotic-associated diarrhoea (Bartlett and Gerding, 2008). CDI occurs when patients receive antibiotics, which disrupt and eradicate their normal enteric gut bacteria, allowing the opportunistic pathogen to proliferate and cause disease.

Recurrent CDI is defined as complete resolution of CDI while on appropriate therapy, followed by recurrence of CDI after treatment has been stopped (Bakken, 2009). A patient being treated for a first episode of CDI has a 10-25% chance of developing recurrent CDI and patients who have had one episode of recurrent CDI have a 50-65% chance of developing multiple episodes of recurrent CDI (Bakken, 2009; Huebner and Surawicz, 2006). Risk factors for CDI include medications that disrupt the intestinal microbiota such as antibiotic use and proton pump inhibitors, prolonged hospitalisation, age >65 years, comorbid medical conditions and severe illness (Kim *et al.*, 2012; Potter and Aravinthan, 2012). The existing treatment options for recurrent CDI are somewhat limited (Huebner and Surawicz, 2006).

Several studies in the literature support an association between recurrent CDI and microbial ecosystem collapse,

i.e. there is an inability of certain individuals to 're-establish' their normal bacterial microbiota following, for instance, antibiotic insult (Chang *et al.*, 2008; Khoruts *et al.,* 2010; Tvede and Rask-Madsen, 1989). This is well illustrated by one study that used 16S rRNA analysis to sequence and analyse bacterial DNA from stool samples of 10 patients: 4 patients with CDI, 3 patients with recurrent CDI and 3 healthy controls (Chang *et al.*, 2008). Stool from patients with more than one positive *C. difficile* toxin measurement (i.e. recurrent CDI) displayed a dramatic loss of microbial diversity compared to the other groups. Interestingly, one patient who had suffered one episode of CDI showed unusually low microbial diversity in his stool sample compared to the others. The authors anecdotally mentioned that this patient went on to develop recurrent CDI after the study was closed, again suggesting recurrent CDI may correlate with loss of gut microbial diversity. In addition, the actual composition may be an important factor, as illustrated by a murine study of CDI showing that loss of colonisation resistance was associated with increased numbers of *Proteobacteria* and decreased numbers of *Firmicutes* (Reeves *et al.*, 2011). This has been supported more recently by a human study which showed that composition may be equally as important as overall diversity (Kassam *et al.*, 2012; Petrof *et al.*, in press).

One treatment for recurrent disease that appears highly effective in case studies is faecal bacteriotherapy or faecal microbiota reconstitution ('stool transplant'). This involves taking stool from a healthy 'donor' and then administering the faecal material to the patient. The goal is to re-establish a more normal stool composition and one with more microbial diversity, given the evidence that microbiota disruption is a significant pathophysiologic component of recurrent CDI. Faecal bacteriotherapy as a treatment for CDI is not a new concept; it was successfully used to treat CDI as early as 1958 (Eiseman *et al.*, 1958). However, it is only in more recent years that this method of treating recurrent CDI has been resurrected, as other conventional therapies have failed. A recent Canadian meeting on faecal transplant therapy revealed that faecal bacteriotherapy treatments of one form or another have been performed in at least 6 major cities in Canada; the attendees at the meeting had collectively performed over 220 faecal bacteriotherapy transplants in total, with cure rates over 90% (Allen-Vercoe *et al.*, 2012). A systematic review of recurrent *C. difficile* cases treated with faecal bacteriotherapy found that 92% of patients experienced resolution of their symptoms and 89% experienced resolution after a single treatment; 4% experienced a relapse in symptoms after faecal bacteriotherapy and resolution rate was lowest with delivery via the upper gastro-intestinal tract (GIT) (76%) (Gough *et al.*, 2011). Another recent review of the published literature that included 31 studies of patients with (mostly recurrent) CDI who had received faecal bacteriotherapy (total of 376 patients) reported an average cure rate of 90% (range of 65-100%) after one or more infusions. The exact route for treatment was not specified in all studies, but the majority of patients (>87%) received the faecal therapy via the lower GIT either by rectal enema or delivery by colonoscopy. Some received it through a nasogastric tube, a gastrostoma or a jejunal infusion. Many studies, but not all, chose donors that were related to the patient (Borody and Khoruts, 2012). Finally, a more recent systematic review using more rigorous inclusion criteria included only 7 trials (total of 124 patients) from 2000 to October 2011 and found an overall cure rate of 83% (Guo *et al.*, 2012). No data are available about the bacteria present in the donor stool samples and other than screening for conventional pathogens, the intestinal microbiota in these studies were not analysed for content prior to the faecal infusions.

The optimal combination(s) of bacterial strains that would be necessary to restore the microbial balance to treat recurrent CDI remains largely unexplored and undefined. In an attempt to investigate this issue, one small study used culture-independent techniques in a patient with recurrent CDI who received faecal microbial reconstitution (Khoruts *et al.*, 2010). Using 16S rRNA gene analysis and bacterial DNA sequencing, it was shown that the recipient patient had less of certain species (e.g. *Bacteroides* spp.) than the donor, but the recipient had more of some other bacterial genera (*Veillonella, Clostridium, Lactobacillus, Streptococcus,* and some unclassified bacteria) not present in the donor. However, at day 14 post-procedure, the patient's diarrhoea had resolved and the patient's intestinal microbiota began to resemble the composition of the donor with a predominance of *Bacteroides* spp. By day 33, bacterial diversity increased as several other predominantly butyrate-producing groups of anaerobic bacteria (*Ruminococcaceae, Anaerostipes,* other *Firmicutes*) became abundant (also present in donor) and the patient experienced no further episodes of CDI. This study was the first of its kind to use molecular approaches to characterise differences in microbial populations pre- and post-transplantation of faecal microbial therapy and to document the ability of the donor bacteria to 'colonise' the recipient. It also supported the results of an earlier study of 6 patients with recurrent CDI treated with either faecal bacteriotherapy or bacterial enemas, which found that *Bacteroides* species were universally absent during the patients' illness but were present in large numbers after bacteriotherapy/resolution of symptoms (Tvede and Rask-Madsen, 1989).

Despite its enormous potential, there are some major challenges in using faecal microbial therapy. Firstly, although donors are screened as close as possible to the time that they provide their donation, there is a window of time between sampling and donation during which donors could possibly become infected with a pathogen. If asymptomatic, donors could unwittingly pass on an infection to the patient

through stool donation. Secondly, donors may harbour opportunistic pathogens that, while not causing disease in the donor, may be problematic for a recipient patient, particularly if that patient is immunocompromised. A better working knowledge of the exact composition of the ecosystem being administered may thus enhance overall safety of this approach.

3. Making it better: synthetic stool/purified bacterial ecosystem approaches

As mentioned earlier, in 1989, Tvede and Rask-Madsen reported the use of a consortium of ten cultured bacterial species, originally isolated from human faeces, to treat refractive CDI in six patients (Tvede and Rask-Madsen, 1989). The consortium consisted of both facultative and strict anaerobes and each was cultured axenically before instilling a mix of cultures rectally into each patient as an enema in a saline carrier. This landmark study was notable for its success, as all patients rapidly became asymptomatic within 24 h and subsequent *C. difficile* toxin testing was negative. Despite this success, the treatment modality was not developed for mainstream medicine, perhaps because at the time recurrent CDI was unusual and antibiotic therapy was adequate for clearing infection. Because faecal transplantation has now re-emerged as an effective treatment modality, the natural progression from this point is to apply Tvede and Rask-Madsen's approach to create 'synthetic stool' formulations or stool substitutes that mitigate most, if not all the concerns of using stool in a therapeutic manner. Research in our laboratories has recently taken the first steps towards this goal (Petrof *et al.*, in press).

Microbial Ecosystem Therapeutics

Our approach to MET began with consideration for the selection of a suitable microbiota. In general, the tactic for producing a therapeutic probiotic mixture has relied on combining several microbial species and empirically defining the optimal formulation. Such a formulation may encompass bacterial species derived from different niches or hosts and as such little thought is put into the intrinsic needs of the ecosystem itself. It is known that microbial species within many ecosystems work together to generate stability and this is equally true in the gut ecosystem (Blaut, 2012; Flint *et al.*, 2007; Van den Abbeele *et al.*, 2011). Mindful of this, we allowed Nature to preselect our ecosystem resource by identifying a single, healthy donor from whom to isolate bacterial species for use in a therapeutic ecosystem. By doing so, we ensured that any strains selected for ultimate therapeutic use would be compatible: at worst capable of co-existing and at best able to impart a synergistic beneficial effect. Our donor was selected based on optimal health parameters including average BMI, non-smoker, no history of chronic disease and, perhaps most importantly, minimal to no exposure to antibiotics.

One of the perceived difficulties of the use of a cultured microbial ecosystem from the human gut is that the gut microbiota has traditionally been considered as 'unculturable' with only ~20% of the resident microbes being amenable to axenic culture (Eckburg *et al.*, 2005; Hold *et al.*, 2002; Suau *et al.*, 1999). However, this widely promoted figure is likely to be a gross underestimate of the culturable species present in this niche (Duncan *et al.*, 2007). There is no doubt that, being for the most part strictly anaerobic and nutritionally fastidious, many gut microbes are out-of-reach to most microbiology laboratories. However, recent technological advances as well as a growing interest in the human gut ecosystem have led to a surge of progress in this area. Thus, the human gut microbiota needs no longer be considered to be inaccessible and the term 'unculturable' should be replaced with 'as-yet uncultured'.

We were able to culture over 60 bacterial species from our selected donor, of which 33 were chosen based on favourable antibiotic resistance profiles and reliable culture. The resulting ecosystem 'MET' was formulated by combining cultures to a predetermined ratio in a saline base and was administered via colonoscopy to 2 patients suffering from severe, refractive CDI, resulting in clinical cure in both cases. Both patients remained well at their 6-month follow-up assessment. Sequence analysis of the microbial diversity of stool samples taken just prior to and for intervals to 6 months after the introduction of MET demonstrated that patient gut microbiota profiles shifted significantly after treatment, corresponding with cure, and that many members of the MET formulation persisted in the patients' microbiota, despite the fact that the therapeutic was administered only once in each case. Also of note, both patients subsequently received multiple courses of antibiotics for reasons unrelated to their CDI and despite this antimicrobial onslaught neither of them experienced any relapse of CDI (Petrof *et al.*, in press). These findings suggest that the use of a probiotic 'ecosystem therapeutic' is fundamentally different to the use of more traditional single-species or simple mixture probiotics with respect to both effectiveness and persistence (Table 1). Hence, MET represents a prototype therapeutic ecosystem and demonstrates the principle that ecosystem therapeutics is an effective and safe approach to treatment of CDI.

The next step, currently under development in our laboratories, is to further optimise a therapeutic ecosystem. Issues to consider include the use of next-generation sequencing techniques to profile the therapeutic formulation metagenomically and transcriptomically, to ensure a balance of biochemical pathways are present that will complement those of the host. This should, of course, include a consideration of the host's usual diet and perhaps

Table 1. Comparison of considerations for different therapeutic approaches using live microorganisms.

	Faecal bacteriotherapy	Probiotic preparations	'Synthetic stool' (cultured gut microbial components)
Reproducibility	Minimal. Relies on donor being repeatedly available. Donor stool may be frozen but efficacy may be affected.	Excellent. Usually small numbers of strains within a formulation, so easily reproduced.	Moderate. Although use of e.g. chemostat batch culture can maximise reproducibility.
Availability	Fair. Stool is readily available but donor screening faecal bacteriotherapy protocols can make approach inaccessible.	Good. Usually produced in bulk and easily obtainable without prescription. Can be expensive.	Good. There is potential to create therapeutic ecosystems in convenient delivery modes similar to probiotics (e.g. oral capsules). However, their use should be restricted by clinicians (prescription only).
Safety	Good. Required extensive screening of donor, but no guarantees that potential pathogens will not be accidentally administered.	Excellent. Safety dossiers required for marketability of probiotics are extensive. Probiotic organisms are by definition GRAS.	Very good. Ecosystem is defined, and thus each component organism can be profiled. May require extra consideration of component species alone and as a community.
Controllability	Poor. Once instilled, cannot be easily monitored or removed.	Excellent. Can be monitored and will naturally be expelled by the resident microbiota.	Excellent. Can be monitored and although it may persist, knowledge of antibiotic profiles will facilitate removal if necessary.
Stability	Poor. Usually stool needs to be processed and instilled to the patient within a few hours of donation.	Excellent. Industrial processes have optimised shelf-life of many probiotic formulations.	Excellent. In principle the same industrial processes used for probiotic preservation can be applied to ecosystems.
Palatability	Poor. Usually a last resort for patients because of unpleasantness of procedure for patient and care-givers, and embarrassment for donor.	Excellent. High safety profile and convenient dosage forms. No association with faecal matter.	Very good. Convenient dosage forms can be developed as for probiotics. May be psychologically associated with faecal microbiota by some patients.
Effectiveness	Very good. Shows much promise as an effective therapy.	Poor. Only low to moderate effectiveness reported.	Very good. Evidence for effectiveness similar to that of faecal transplant (Petrof *et al*., in press).

also the definition of host enterotype as defined by their long-term dietary patterns (Arumugam *et al.*, 2011; Wu *et al.*, 2011).

The issue of product stabilisation and delivery is also important to address. We suggest that therapeutic ecosystems possess enough stability that, given the correct environmental conditions, they should be able to be continuously cultured as entire ecosystems using, for example, a chemostat system. We have already demonstrated that MET can exist at steady state in a chemostat (Allen-Vercoe, unpublished data). This approach would have several key benefits. Firstly, the ecosystem would be readily available when required, through the simple drawing off of dosages from an active chemostat. Secondly, each dose could be supplied with its own bolus of nutrients, allowing for optimal conditions for establishment of the community into a bowel that may be depleted in nutrient availability (either through bowel prep usage or chronic diarrhoea). Finally, each dose drawn from a chemostat at 'steady state' would come with the component species already in balance in terms of relative abundance, minimising preparative effort needed to reproduce and optimal formulation. Initial data from our laboratories has suggested that, at least in one patient, the abundance profile of MET generated in a chemostat at steady state broadly matched that obtained in the patient stool sample 2 weeks after MET administration (Figure 2). The factors governing gut microbiota balance in a human host have not yet been completely elucidated, but as a side benefit, study of defined microbial communities such as MET in patient populations will give enormous insight into this aspect of human microbial ecology.

4. Other specific diseases where microbial ecosystems therapeutics might be a useful strategy

Ulcerative colitis

When compared to the microbiota of healthy patients undergoing routine colon cancer screening procedures, the microbiota from patients with ulcerative colitis (UC) displays marked differences (Qin *et al.*, 2010). A reduction in the richness, evenness and overall diversity of the microbiota has been reported in children and adolescents with UC compared to either healthy controls or siblings (Andoh *et al.*, 2011; Michail *et al.*, 2011; Nemoto *et al.*, 2012; Noor *et al.*, 2010). Faecal samples from 27 inpatient children with severe UC were compared to faecal samples from 26 healthy controls as part of a prospective multicenter

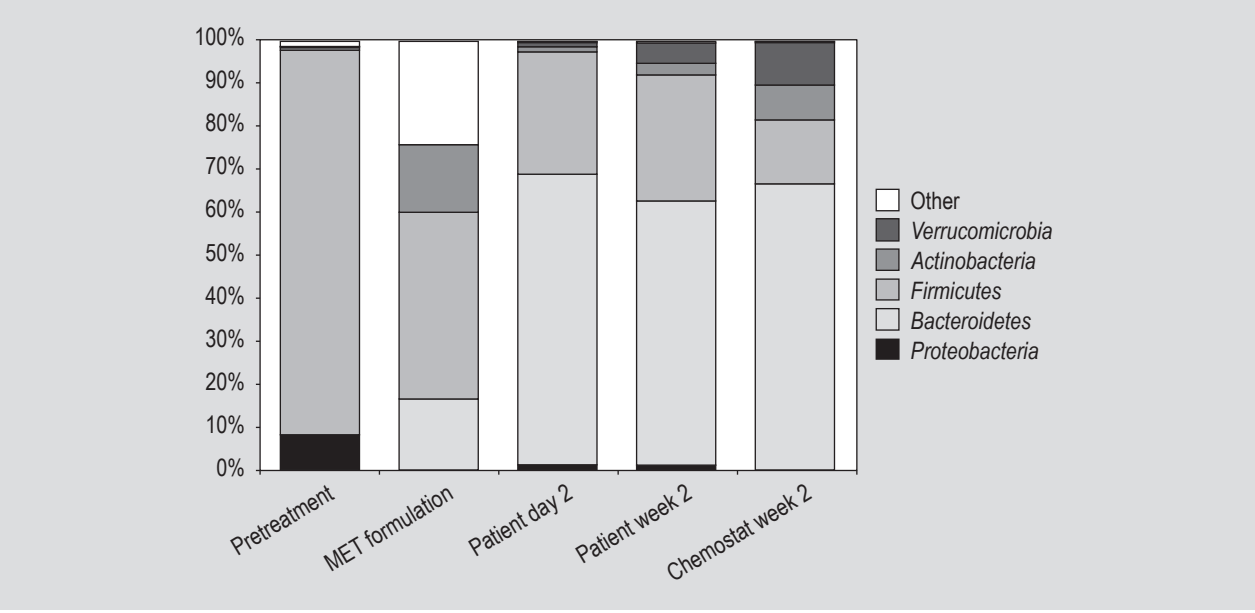

Figure 2. Barplot to show bacterial abundance at the family level from stool samples and chemostat effluent following MET administration in one patient (Petrof *et al.*, 2012). A MET consisting of 33 bacterial species isolated from a single healthy donor was instilled via colonoscopy into a patient with severe, refractory *C. difficile* infection. At the same time as instilling the MET into the patient, we also seeded a chemostat culture with the formulation and allowed it to reach steady state under conditions mimicking that of the human distal colon (pH 7, 37 °C, 24 h retention time and a nutrient source rich in mucin and resistant starch substrates).

study and overall diversity of the microbiota as well as the number of microbial phylospecies were reduced in UC patients vs. controls (Michail *et al.*, 2011). There was also a notable decrease in *Clostridium* species but an increase in *Gammaproteobacteria*.

Another study (Lepage *et al.*, 2011), which investigated differences between healthy twins and UC patients, similarly showed that the patients with UC possessed less biodiversity than their healthy twins. The healthy siblings possessed higher percentages of bacteria from the *Lachnospiraceae* and *Ruminococcaceae* groups. In contrast, others have found that there is an increase in *Bacteroides* and *Prevotella* spp. in colonic mucosa of patients with UC (Lucke *et al.*, 2006). Since organisms such as *Bacteroides* and *Prevotella* produce sulphatases, which are capable of degrading mucin (Tsai *et al.*, 1992; Wright *et al.*, 2000), it is plausible that these bacterial communities may play a role in the pathogenesis of UC by interfering with normal barrier function and affecting the integrity of the mucosal lining of the intestinal epithelium. The observation that increased levels of mucin-degrading sulphatases have been described in active UC patients supports this possibility (Tsai *et al.*, 1995).

These interesting observations of the microbiome of UC patients have led some to propose that intentional manipulation of the microbiome may provide some additional novel therapeutic options (Talley *et al.*, 2011). Some published data on the use of faecal microbial therapy in treating UC exists, suggesting that replacement of a damaged microbial ecosystem with a healthier community of intestinal bacteria may be a feasible treatment option. One published report describes six patients with confirmed diagnosis of UC who responded to a 5-day course of daily faecal enemas (Borody *et al.*, 2003). However, one of the authors commented that (unlike CDI) multiple recurrent infusions are usually required to achieve durable remission (Borody and Campbell, 2011), suggesting that faecal transplant therapy may not be as effective for inflammatory bowel disease. Hence, it is tempting to speculate that a 'synthetic stool' approach, wherein defined microbial ecosystems are used to replace the damaged or dysfunctional microbial communities in the UC intestine, may provide a novel approach to complement the currently available immunomodulating therapies targeting the host immune response. Before this can be accomplished, however, it still needs to be established whether the microbial changes in patients with UC are a contributing cause or merely an effect of the disease. Although many associative reports have been published (Gosiewski *et al.*, 2012; Lepage *et al.*, 2011; Michail *et al.*, 2011), the exact etiologic role of changes described in the microbiota of patients with UC still remains poorly understood; several studies are underway to further explore and define this area.

Obesity

Obesity is a complex disorder characterised by accumulation of adipose tissue. Although once simply thought of as a mere imbalance between caloric intake and expenditure, now it is recognised that additional psychological and environmental factors can contribute to a predisposition for the obese state (Anonymous, 2012; Harris *et al.*, 2012). The involvement of the gut microbiota in obesity is one of the environmental factors that has recently come under scrutiny (Frazier *et al.*, 2011; Greenblum *et al.*, 2012; Harris *et al.*, 2012; Kaplan and Walker, 2012; Thompson, 2012).

There have been several studies to date that have attempted to compare the gut microbiota of lean and obese people in order to define an obese microbiota profile. At the phylum level, contradictory results from different groups have made it difficult to ascertain an obesity signature (Duncan *et al.*, 2008; Jumpertz *et al.*, 2011; Ley *et al.*, 2005, 2006b; Schwiertz *et al.*, 2010; Turnbaugh *et al.*, 2006). However, more recently Greenblum *et al.* (2012) have used a metagenomic systems biology approach to show that lean and obese microbiomes do indeed differ and that these differences are related to the metabolic pathways encoded by the microbiota, with the obese phenotype enriched in pathways likely to interface with the host and additionally reduced in modularity, possibly as a result of reduced diversity.

Turnbaugh *et al*. (2006) showed that colonisation of germ-free mice with microbiota associated with obese mice resulted in greater weight gain in these animals than in germ-free mice colonised with a control microbiota sourced from lean mice. This suggests that the microbiota associated with the obese phenotype had a greater capacity to extract energy from the diet, making it more available to the host. If this is the case, then there is potential to modulate the gut microbiota of obese individuals to reduce the capacity for their microbiota to efficiently extract calories from dietary sources, in turn aiding weight reduction efforts. Faecal transplantation from a lean to an obese person may be the easiest way to modulate the microbiota to this end and recently Vrieze *et al*. (2012) described a clinical trial of faecal transfer from lean to obese individuals resulting in an increase in insulin sensitivity in the recipients. New knowledge such as the metagenome systems biology described above may pave the way to more targeted, safer approaches to the problem; if the foundation for the obese phenotype lies in reduced diversity (Greenblum *et al.*, 2012), for example, it may be possible to evaluate the gut microbial ecosystem of an obese individual and to supply particular taxa (possessing particular, missing metabolic potential) to 'patch up' the dysbiotic microbial community and restore balance to the ecosystem. Although not practically possible at present, knowledge and technology are rapidly advancing to the point where such personalised medicine will become mainstream in the not-too-distant future.

Necrotising enterocolitis

In the USA, 12% of infants xare born prior to 37 weeks gestational age or preterm. These infants represent a unique subset that completes host development concurrent with microbiota colonisation rather than within the normal sterile confines of the maternal uterus. Necrotising enterocolitis (NEC) is an inflammatory bowel necrosis that affects this population, occurring in approximately 10% of premature infants born at less than 1,500 g (Kosloske, 1994; Lemons *et al.*, 2001; MacKendrick and Caplan, 1993; Rayyis *et al.*, 1999; Stoll *et al.*, 2010). Susceptibility to NEC appears inversely related to gestational age, thus, the more immature the infant the more likely it is to get this disease.

Among the primary risk factors for NEC are bacterial colonisation and prematurity. Specifically, preterm infants are often colonised within the neonatal intensive care unit (NICU) and influenced by a hospital environment with exposure to nosocomial pathogens and therapeutic interventions such as broad spectrum antibiotics. Thus while a wide range of aerobic and anaerobic microbiota colonises full-term infants by 10 days of age, studies have shown that by comparison preterm infants in the NICU undergo delayed colonisation with a limited number of bacterial species that tend to be opportunistic pathogens (Goldmann *et al.*, 1978; Schwiertz *et al.*, 2003). Studies have shown that a premature infant has a potentially more pathogenic bacterial balance and an immature gut defence to shield against these bacteria, which likely results in an exaggerated intestinal inflammatory response (Claud and Walker, 2001; Claud *et al.*, 2007).

Evidence suggests that bacterial colonisation patterns are important in the pathogenesis of NEC and preterm infants of mothers receiving broad-spectrum antibiotics prenatally or preterm infants receiving antibiotics postnatally are at higher risk for NEC (Cotten *et al.*, 2009; Kenyon *et al.*, 2001; Wang *et al.*, 2009). Isolated studies have demonstrated associations with organisms including *Enterobacteriaceae* (Bell *et al.,* 1979*;* Millar *et al.,* 1992)*,* delta toxin positive methicillin-resistant *Staphylococcus aureus* (Overturf *et al.,* 1990) and *Clostridium* spp. (Sturm *et al.,* 1980). Sequencing-based studies find that the microbial community structure in NEC patients is distinct based on a decrease in diversity, an increase in abundance of *Gammaproteobacteria* and a decrease in other bacterial species (Wang *et al.*, 2009), but no specific pathogen has been recognised as causal.

Although no causal pathogen has been identified, there is some evidence that microbial colonisation with the appropriate non-pathogenic organisms may promote health of the preterm gut so that it is protected and thus less susceptible to NEC. The probiotic literature lends some support to this concept. Studies using live *Bifidobacterium* in a rat model of NEC have demonstrated

decreased incidence of NEC associated with reduction of inflammatory reaction in the ileum, regulation of main components of mucus layer and improvement of intestinal integrity (Caplan *et al.*, 1999; Khailova *et al.*, 2009). One study that utilised *Bifidobacterium breve* to prevent mucositis in children undergoing chemotherapy and to prevent NEC in premature infants noted an enhanced colonisation of anaerobes in the treatment group receiving the probiotic (Yamashiro and Nagata, 2010). Clinical trials using mixtures of probiotic organisms such as Infloran® (*Lactobacillus acidophilus* and *Bifidobacterium infantis*) (Hoyos, 1999; Lin *et al.*, 2005, 2008) and ABC Dophilus® (*B. infantis*, *Streptococcus thermophilus* and *Bifidobacterium bifidus* (Bin-Nun *et al.*, 2005)) have also shown protection against NEC, further confirming the potential of microbial therapeutics for this disease.

A key question for NEC has always been why some premature infants get NEC while others are protected. Perhaps the preterm infants to be studied are not those that develop NEC but those that remain healthy to identify the components of a gut microbial ecosystem associated with health. Studying the composition and function of the microbial ecosystems of healthy preterm infants may lead to improved understanding of the essential components of the preterm infant gut microbiota and lead to development of ecosystem therapeutics specifically for this patient population to prevent NEC, but also to ensure long-term health.

Regressive-type autism

The gut-brain-microbiota axis is a relatively new term coined to describe the increasingly clear interactions between these three systems (Forsythe and Kunze, 2012). Whilst the gut-microbiota connection was an obvious one, the now well-established link between the microbiota and the brain has come as somewhat of a surprise. Much of what is known about the gut-brain-microbiota axis has been discovered through the study of germ-free (GF) animals, which are raised in a sterile environment and have a plethora of physiological abnormalities as a result (Wagner, 2008). Whilst many of these abnormalities relate to the ability of the microbiota to provide energy and vitamins to the host and to prime the immune system effectively (Wagner, 2008), a role for the microbiota in the development of the hypothalamic-pituitary-adrenal (HPA) axis was demonstrated in 2004 by Sudo *et al.* who implemented mild restraint stress in GF mice and showed that these animals released larger amounts of corticosterone and adrenocorticotrophin hormone compared to control animals possessing a specific pathogen free microbiota (Sudo *et al.*, 2004). These researchers went on to show that restoration of the gut microbiota in GF animals resulted in normal levels of secretion of these critical stress response hormones. However, this effect was dependent on the time frame during early development in which the microbiota was restored, indicating a developmental window of time in which HPA axis and microbiota communicate to 'set' an appropriate response that continues through life (Sudo *et al.*, 2004). Induced gut microbial dysbiosis has been shown to alter the behaviour of mice in a variety of tests designed to measure anxiety-like responses. Both antibiotic use and deliberate infection with pathogens such as *Citrobacter rodentium* have been shown to modulate behaviour, with the former approach giving rise to a less anxious phenotype (Bercik *et al.*, 2011) and the latter approach resulting in enhanced anxiety and stress-induced memory dysfunction (Gareau *et al.*, 2011). Although the mechanisms that link gut microbiota disturbance to behaviour are multifactorial and not yet completely defined, recently the vagus nerve has been demonstrated as a critical communication conduit between gut microbes and the brain (Bravo *et al.*, 2011).

With this microbiota-brain connection in mind the potential for some psychiatric diseases to be viewed in a totally new light has recently arisen. One such disease, regressive type autism, is a pervasive developmental disorder that becomes evident within the first 3 years of life and is characterised by an abnormal development of social and communication skills in an affected child following a period of apparently normal progression (Finegold *et al.*, 2012; Stefanatos, 2008). Interestingly, gastrointestinal disturbances are frequently co-reported in affected children (Parracho *et al.*, 2005), as are altered urinary metabolite phenotypes, characteristic of metabolites originating from the gut microbiota (Clayton, 2012; Yap *et al.*, 2010). MacFabe and colleagues have clearly shown an association between the enteric bacterial fermentation product propionic acid and autistic behaviour in a rat model. They hypothesise that an excess of propionic acid resulting from gut dysbiosis and resultant excessive fermentation by certain members of the gut microbiota may be at the source of this (MacFabe *et al.*, 2011; Thomas *et al.*, 2012). Several independent studies done to characterise the gut microbiota of affected children have shown apparent dysbioses compared to age-matched controls, including over-representation of *Clostridium* spp. (Finegold *et al.*, 2010; Parracho *et al.*, 2005), *Desulfovibrio* spp. (Finegold, 2011a) and *Sutterella* spp. (Williams *et al.*, 2012), and under-representation of *Akkermansia muciniphila* and *Bifidobacterium* spp. (Wang *et al.*, 2011), the significance of which has yet to be defined. Potentially, over-abundant microbes could represent novel pathogens and theories for e.g. bacterial toxin-mediated disease have been proposed (Bolte, 1998; Finegold, 2011b). Late-onset autism is hypothesised to be caused by early-life exposure to antibiotic agents during a critical window of childhood development. Comparisons have been drawn to similar antibiotic-associated infections such as CDI and one small trial of the use of oral vancomycin in a group of severely autistic children reported impressive developmental gains during treatment that diminished

when the drug was withdrawn (Sandler *et al.*, 2000). The phenomenon of vancomycin-induced improvement followed by relapse on drug withdrawal is well-known to infectious disease physicians who treat CDI and is related to the persistence of the spore-forming *C. difficile* during treatment (Gerding *et al.*, 2008). Whilst a role for an infectious agent in regressive autism has yet to be conclusively demonstrated, parents of affected children often report benefits of strict dietary intervention (and as a result, likely gut microbiota modulation) on behaviour (Pennesi and Klein, 2012; Reichelt and Knivsberg, 2009).

Given the increasing evidence for the involvement of the gut microbiota in regressive autism, an intriguing possibility emerges that ecosystem therapeutics may be of benefit in this patient population. Drawing the analogy to CDI, if this type of autism is indeed directly related to gut microbial dysbiosis and/or infection with a specific, as yet unknown pathogen, displacement of the dysbiotic ecosystem/pathogen using a well-designed synthetic microbiota specifically targeted to this paediatric population may represent a plausible treatment for this devastating disease. Further work is urgently needed to define the functional dysbiosis of the gut microbiota of regressive autistics and to design ecosystem therapeutics specifically targeted to safely and effectively correct any deficits. Whether this targeted therapy will work to alleviate the social and communication problems experienced by these children remains to be seen, but seems increasingly plausible given the rapidly growing body of evidence for the importance of the gut-brain-microbiota axis.

5. Summary and conclusions

The more we discover about the gut microbiota, the more we understand that microbial ecosystems play a crucial role in maintaining human health (The Human Microbiome Project Consortium, 2012). With the changes imposed by advances in human civilisation (e.g. widespread use of broad-spectrum antibiotics, use of low-dose antimicrobials in the food supply and in soaps and other cleaning agents), we are witnessing unprecedented changes in these microbiologic ecosystems. We are not only witnessing a loss in horizontal transmission (e.g. clean water, better overall hygiene), but also a loss of vertical transmission (e.g. increased numbers of Caesarean sections, decreased emphasis on breastfeeding) and because of this dual loss there is slim chance these microbial ecosystems will ever fully recover: these highly susceptible intestinal ecosystems are slowly being wiped out over time. In the void which is left behind, new organisms will invariably replace the old ones and with this comes the inherent risk that opportunistic pathogens will fill the empty niche. Thus it seems logical to strive to harness the microbiota of supremely healthy people to replace dysfunctional, damaged or diseased microbiota in others. However, many questions still remain to be answered before this approach can become mainstream. In order to push the field forward, we propose several questions on which future research should focus:

- How do we define a healthy gut microbiota?
- How does ecosystem replacement occur and what happens to the displaced microbiota?
- What are the implications of prophylactic use of ecosystem therapeutics (for example, when used to administer to patients at risk of developing *C. difficile* colitis during a stay in hospital).
- What are the long-term effects of ecosystem therapeutics (e.g. how do we ensure a microbe with potentially pathogenic properties is not transferred?).
- If gut and brain are as intrinsically linked as it seems, then what are the ethical considerations for ecosystem therapeutics? For example, should the long-term behavioural effects following ecosystem therapeutics be studied?
- What encompasses normal intrinsic variability within an individual's microbiome and how does this vary over time?

By restoring the beneficial microbes and 'lost' microbial communities, a new paradigm in the practice of medicine may be on the horizon that builds on the concept of probiotics, replacing the use of conventional single strain probiotic formulations with large, complex mixtures of intact bacterial communities. The concept of MET would emphasise the rational design of healthy, resilient and robust microbial communities that, in conjunction with intelligent and judicious use of select antimicrobials, could be used to maintain or restore human health. A more holistic understanding of what constitutes gut health will ultimately guide future approaches to correcting gut dysbiosis and the answer surely lies in the consideration of the entire microbial ecosystem rather than its individual components.

References

Allen-Vercoe, E., Reid, G., Viner, N., Gloor, G.B., Hota, S., Kim, P., Lee, C.H., O'Doherty, K., Vanner, S., Weese, J.S. and Petrof, E.O., 2012. A Canadian working group report on fecal microbial therapy: microbial ecosystems therapeutics. Canadian Journal of Gastroenterology 26: 457-462.

Andoh, A., Imaeda, H., Aomatsu, T., Inatomi, O., Bamba, S., Sasaki, M., Saito, Y., Tsujikawa, T. and Fujiyama, Y., 2011. Comparison of the fecal microbiota profiles between ulcerative colitis and Crohn's disease using terminal restriction fragment length polymorphism analysis. Journal of Gastroenterology 46: 479-486.

Anonymous, 2012. Obesity and overweight factsheet. World Health Organization. Available at: http://www.who.int/mediacentre/factsheets/fs311/en/.

Arumugam, M., Raes, J., Pelletier, E., Le Paslier, D., Yamada, T., Mende, D.R., Fernandes, G.R., Tap, J., Bruls, T., Batto, J.M., Bertalan, M., Borruel, N., Casellas, F., Fernandez, L., Gautier, L., Hansen, T., Hattori, M., Hayashi, T., Kleerebezem, M., Kurokawa, K., Leclerc, M., Levenez, F., Manichanh, C., Nielsen, H.B., Nielsen, T., Pons, N., Poulain, J., Qin, J., Sicheritz-Ponten, T., Tims, S., Torrents, D., Ugarte, E., Zoetendal, E.G., Wang, J., Guarner, F., Pedersen, O., De Vos, W.M., Brunak, S., Dore, J., Antolin, M., Artiguenave, F., Blottiere, H.M., Almeida, M., Brechot, C., Cara, C., Chervaux, C., Cultrone, A., Delorme, C., Denariaz, G., Dervyn, R., Foerstner, K.U., Friss, C., Van de Guchte, M., Guedon, E., Haimet, F., Huber, W., Van Hylckama-Vlieg, J., Jamet, A., Juste, C., Kaci, G., Knol, J., Lakhdari, O., Layec, S., Le Roux, K., Maguin, E., Merieux, A., Melo Minardi, R., M'Rini, C., Muller, J., Oozeer, R., Parkhill, J., Renault, P., Rescigno, M., Sanchez, N., Sunagawa, S., Torrejon, A., Turner, K., Vandemeulebrouck, G., Varela, E., Winogradsky, Y., Zeller, G., Weissenbach, J., Ehrlich, S.D. and Bork, P., 2011. Enterotypes of the human gut microbiome. Nature 473: 174-180.

Bakken, J.S., 2009. Fecal bacteriotherapy for recurrent *Clostridium difficile* infection. Anaerobe 15: 285-289.

Bartlett, J.G. and Gerding, D.N., 2008. Clinical recognition and diagnosis of *Clostridium difficile* infection. Clinical Infectious Diseases 46 Suppl. 1: S12-S18.

Bell, M.J., Shackelford, P.G., Feigin, R.D., Ternberg, J.L. and Brotherton, T., 1979. Alterations in gastrointestinal microbiota during antimicrobial therapy for necrotizing enterocolitis. Pediatrics 63: 425-428.

Bercik, P., Denou, E., Collins, J., Jackson, W., Lu, J., Jury, J., Deng, Y., Blennerhassett, P., Macri, J., McCoy, K.D., Verdu, E.F. and Collins, S.M., 2011. The intestinal microbiota affect central levels of brain-derived neurotropic factor and behavior in mice. Gastroenterology 141: 599-609.

Bin-Nun, A., Bromiker, R., Wilschanski, M., Kaplan, M., Rudensky, B., Caplan, M. and Hammerman, C., 2005. Oral probiotics prevent necrotizing enterocolitis in very low birth weight neonates. Journal of Pediatrics 147: 192-196.

Blaut, M., 2012. Ecology and physiology of the intestinal tract. Current Topics in Microbiology and Immunology, in press. DOI: http://dx.doi.org/10.1007/82_2011_192.

Bolte, E.R., 1998. Autism and *Clostridium tetani*. Medical Hypotheses 51: 133-144.

Borody, T.J. and Campbell, J., 2011. Fecal microbiota transplantation: current status and future directions. Expert Reviews in Gastroenterology and Hepatology 5: 653-655.

Borody, T.J. and Khoruts, A., 2012. Fecal microbiota transplantation and emerging applications. Nature Reviews Gastroenterology and Hepatology 9: 88-96.

Borody, T.J., Warren, E.F., Leis, S., Surace, R. and Ashman, O., 2003. Treatment of ulcerative colitis using fecal bacteriotherapy. Journal of Clinical Gastroenterology 37: 42-47.

Bravo, J.A., Forsythe, P., Chew, M.V., Escaravage, E., Savignac, H.M., Dinan, T.G., Bienenstock, J. and Cryan, J.F., 2011. Ingestion of *Lactobacillus* strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proceedings of the National Academy of Sciences of the USA 108: 16050-16055.

Caplan, M.S., Miller-Catchpole, R., Kaup, S., Russell, T., Lickerman, M., Amer, M., Xiao, Y. and Thomson Jr., R., 1999. Bifidobacterial supplementation reduces the incidence of necrotizing enterocolitis in a neonatal rat model. Gastroenterology 117: 577-583.

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

Claud, E.C. and Walker, W.A., 2001. Hypothesis: inappropriate colonization of the premature intestine can cause neonatal necrotizing enterocolitis. The FASEB Journal 15: 1398-1403.

Claud, E.C., Zhang, X., Petrof, E.O. and Sun, J., 2007. Developmentally regulated tumor necrosis factor-alpha induced nuclear factor-kappaB activation in intestinal epithelium. American Journal of Physiology – Gastrointestinal and Liver Physiology 292: G1411-G1419.

Clayton, T.A., 2012. Metabolic differences underlying two distinct rat urinary phenotypes, a suggested role for gut microbial metabolism of phenylalanine and a possible connection to autism. FEBS Letters 586: 956-961.

Cotten, C.M., Taylor, S., Stoll, B., Goldberg, R.N., Hansen, N.I., Sanchez, P.J., Ambalavanan, N. and Benjamin Jr., D.K., 2009. Prolonged duration of initial empirical antibiotic treatment is associated with increased rates of necrotizing enterocolitis and death for extremely low birth weight infants. Pediatrics 123: 58-66.

Duncan, S.H., Lobley, G.E., Holtrop, G., Ince, J., Johnstone, A.M., Louis, P. and Flint, H.J., 2008. Human colonic microbiota associated with diet, obesity and weight loss. International Journal of Obesity 32: 1720-1724.

Duncan, S.H., Louis, P. and Flint, H.J., 2007. Cultivable bacterial diversity from the human colon. Letters in Applied Microbiology 44: 343-350.

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., 2005. Diversity of the human intestinal microbial flora. Science 308: 1635-1638.

Eiseman, B., Silen, W., Bascom, G.S. and Kauvar, A.J., 1958. Fecal enema as an adjunct in the treatment of pseudomembranous enterocolitis. Surgery 44: 854-859.

Finegold, S.M., 2011a. *Desulfovibrio* species are potentially important in regressive autism. Medical Hypotheses 77: 270-274.

Finegold, S.M., 2011b. State of the art; microbiology in health and disease. Intestinal bacterial flora in autism. Anaerobe 17: 367-368.

Finegold, S.M., Dowd, S.E., Gontcharova, V., Liu, C., Henley, K.E., Wolcott, R.D., Youn, E., Summanen, P.H., Granpeesheh, D., Dixon, D., Liu, M., Molitoris, D.R. and Green 3rd, J.A., 2010. Pyrosequencing study of fecal microbiota of autistic and control children. Anaerobe 16: 444-453.

Finegold, S.M., Downes, J. and Summanen, P.H., 2012. Microbiology of regressive autism. Anaerobe 18: 260-262.

Flint, H.J., Duncan, S.H., Scott, K.P. and Louis, P., 2007. Interactions and competition within the microbial community of the human colon: links between diet and health. Environmental Microbiology 9: 1101-1111.

Forsythe, P. and Kunze, W.A., 2012. Voices from within: gut microbes and the CNS. Cellular and Molecular Life Sciences, in press. DOI: http://dx.doi.org/10.1007/s00018-012-1028-z.

Frazier, T.H., DiBaise, J.K. and McClain, C.J., 2011. Gut microbiota, intestinal permeability, obesity-induced inflammation, and liver injury. Journal of Parenteral and Enteral Nutrition 35: 14S-20S.

Gareau, M.G., Wine, E., Rodrigues, D.M., Cho, J.H., Whary, M.T., Philpott, D.J., Macqueen, G. and Sherman, P.M., 2011. Bacterial infection causes stress-induced memory dysfunction in mice. Gut 60: 307-317.

Gerding, D.N., Muto, C.A. and Owens Jr., R.C., 2008. Measures to control and prevent *Clostridium difficile* infection. Clinical Infectious Diseases 46 Suppl. 1: S43-S49.

Goldmann, D.A., Leclair, J. and Macone, A., 1978. Bacterial colonization of neonates admitted to an intensive care environment. Journal of Pediatrics 93: 288-293.

Gosiewski, T., Strus, M., Fyderek, K., Kowalska-Duplaga, K., Wedrychowicz, A., Jedynak-Wasowicz, U., Sladek, M., Pieczarkowski, S., Adamski, P. and Heczko, P.B., 2012. Horizontal distribution of the fecal microbiota in adolescents with inflammatory bowel disease. Journal of Pediatric Gastroenterology and Nutrition 54: 20-27.

Gough, E., Shaikh, H. and Manges, A.R., 2011. Systematic review of intestinal microbiota transplantation (fecal bacteriotherapy) for recurrent *Clostridium difficile* infection. Clinical Infectious Diseases 53: 994-1002.

Greenblum, S., Turnbaugh, P.J. and Borenstein, E., 2012. Metagenomic systems biology of the human gut microbiome reveals topological shifts associated with obesity and inflammatory bowel disease. Proceedings of the National Academy of Sciences of the USA 109: 594-599.

Guo, B., Harstall, C., Louie, T., Veldhuyzen van Zanten, S. and Dieleman, L.A., 2012. Systematic review: faecal transplantation for the treatment of *Clostridium difficile*-associated disease. Alimentary Pharmacology and Therapeutics 35: 865-875.

Harris, K., Kassis, A., Major, G. and Chou, C.J., 2012. Is the gut microbiota a new factor contributing to obesity and its metabolic disorders? Journal of Obesity 2012: 879151.

Hold, G.L., Pryde, S.E., Russell, V.J., Furrie, E. and Flint, H.J., 2002. Assessment of microbial diversity in human colonic samples by 16S rDNA sequence analysis. FEMS Microbiology Ecology 39: 33-39.

Hoyos, A.B., 1999. Reduced incidence of necrotizing enterocolitis associated with enteral administration of *Lactobacillus acidophilus* and *Bifidobacterium infantis* to neonates in an intensive care unit. International Journal of Infectious Diseases 3: 197-202.

Huebner, E.S. and Surawicz, C.M., 2006. Treatment of recurrent *Clostridium difficile* diarrhea. Gastroenterology and Hepatology 2: 203-208.

Jumpertz, R., Le, D.S., Turnbaugh, P.J., Trinidad, C., Bogardus, C., Gordon, J.I. and Krakoff, J., 2011. Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. The American Journal of Clinical Nutrition 94: 58-65.

Kaplan, J.L. and Walker, W.A., 2012. Early gut colonization and subsequent obesity risk. Current Opinion in Clinical Nutrition and Metabolic Care 15: 278-284.

Kassam, Z., Hundal, R., Marshall, J.K. and Lee, C.H., 2012. Fecal transplant via retention enema for refractory or recurrent *Clostridium difficile* infection. Archives of Internal Medicine 172: 191-193.

Kenyon, S.L., Taylor, D.J. and Tarnow-Mordi, W., 2001. Broad-spectrum antibiotics for spontaneous preterm labour: the ORACLE II randomised trial. ORACLE Collaborative Group. The Lancet 357: 989-994.

Khailova, L., Dvorak, K., Arganbright, K.M., Halpern, M.D., Kinouchi, T., Yajima, M. and Dvorak, B., 2009. *Bifidobacterium bifidum* improves intestinal integrity in a rat model of necrotizing enterocolitis. American Journal of Physiology – Gastrointestinal and Liver Physiology 297: G940-G949.

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

Kim, Y.G., Graham, D.Y. and Jang, B.I., 2012. Proton pump inhibitor use and recurrent *Clostridium difficile*-associated disease: a case-control analysis matched by propensity score. Journal of Clinical Gastroenterology 46: 397-400.

Kosloske, A.M., 1994. Epidemiology of necrotizing enterocolitis. Acta Paediatrica. Suppl. 396: 2-7.

Lemons, J.A., Bauer, C.R., Oh, W., Korones, S.B., Papile, L.A., Stoll, B.J., Verter, J., Temprosa, M., Wright, L.L., Ehrenkranz, R.A., Fanaroff, A.A., Stark, A., Carlo, W., Tyson, J.E., Donovan, E.F., Shankaran, S. and Stevenson, D.K., 2001. Very low birth weight outcomes of the National Institute of Child Health and Human Development Neonatal Research N etwork, January 1995 through December 1996. NICHD Neonatal Research Network. Pediatrics 107: E1.

Lepage, P., Hasler, R., Spehlmann, M.E., Rehman, A., Zvirbliene, A., Begun, A., Ott, S., Kupcinskas, L., Dore, J., Raedler, A. and Schreiber, S., 2011. Twin study indicates loss of interaction between microbiota and mucosa of patients with ulcerative colitis. Gastroenterology 141: 227-236.

Ley, R.E., Backhed, F., Turnbaugh, P., Lozupone, C.A., Knight, R.D. and Gordon, J.I., 2005. Obesity alters gut microbial ecology. Proceedings of the National Academy of Sciences of the USA 102: 11070-11075.

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

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

Lin, H.C., Hsu, C.H., Chen, H.L., Chung, M.Y., Hsu, J.F., Lien, R.I., Tsao, L.Y., Chen, C.H. and Su, B.H., 2008. Oral probiotics prevent necrotizing enterocolitis in very low birth weight preterm infants: a multicenter, randomized, controlled trial. Pediatrics 122: 693-700.

Lin, H.C., Su, B.H., Chen, A.C., Lin, T.W., Tsai, C.H., Yeh, T.F. and Oh, W., 2005. Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants. Pediatrics 115: 1-4.

Lucke, K., Miehlke, S., Jacobs, E. and Schuppler, M., 2006. Prevalence of *Bacteroides* and *Prevotella* spp. in ulcerative colitis. Journal of Medical Microbiology 55: 617-624.

MacFabe, D.F., Cain, N.E., Boon, F., Ossenkopp, K.P. and Cain, D.P., 2011. Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: relevance to autism spectrum disorder. Behavioural Brain Research 217: 47-54.

MacKendrick, W. and Caplan, M., 1993. Necrotizing enterocolitis. New thoughts about pathogenesis and potential treatments. Pediatric Clinics of North America 40: 1047-1059.

Michail, S., Durbin, M., Turner, D., Griffiths, A.M., Mack, D.R., Hyams, J., Leleiko, N., Kenche, H., Stolfi, A. and Wine, E., 2011. Alterations in the gut microbiome of children with severe ulcerative colitis. Inflammatory Bowel Diseases, in press. DOI: http://dx.doi.org/10.1002/ibd.22860.

Millar, M.R., MacKay, P., Levene, M., Langdale, V. and Martin, C., 1992. *Enterobacteriaceae* and neonatal necrotising enterocolitis. Archives of Disease in Childhood 67: 53-56.

Nemoto, H., Kataoka, K., Ishikawa, H., Ikata, K., Arimochi, H., Iwasaki, T., Ohnishi, Y., Kuwahara, T. and Yasutomo, K., 2012. Reduced diversity and imbalance of fecal microbiota in patients with ulcerative colitis. Digestive Diseases and Sciences, in press. DOI: http://dx.doi.org/10.1007/s10620-012-2236-y.

Noor, S.O., Ridgway, K., Scovell, L., Kemsley, E.K., Lund, E.K., Jamieson, C., Johnson, I.T. and Narbad, A., 2010. Ulcerative colitis and irritable bowel patients exhibit distinct abnormalities of the gut microbiota. BMC Gastroenterology 10: 134.

Overturf, G.D., Sherman, M.P., Scheifele, D.W. and Wong, L.C., 1990. Neonatal necrotizing enterocolitis associated with delta toxin-producing methicillin-resistant *Staphylococcus aureus*. Pediatric Infectious Disease Journal 9: 88-91.

Parracho, H.M., Bingham, M.O., Gibson, G.R. and McCartney, A.L., 2005. Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. Journal of Medical Microbiology 54: 987-991.

Pennesi, C.M. and Klein, L.C., 2012. Effectiveness of the gluten-free, casein-free diet for children diagnosed with autism spectrum disorder: based on parental report. Nutritional Neuroscience 15: 85-91.

Petrof, E.O., Gloor, G.B., Vanner, S.J., Weese, J.S., Carter, D., Daigneault, M.C., Brown, E.M., Schroeter, K. and Allen-Vercoe, E., in press. Stool substitute transplant therapy for the eradication of C. difficile infection: RePOOPulating the gut. Microbiome, in press.

Potter, V.A. and Aravinthan, A., 2012. Identifying patients at risk of severe *Clostridium difficile*-associated disease. British Journal of Hospital Medicine 73: 265-270.

Qin, J., Li, R., Raes, J., Arumugam, M., Burgdorf, K.S., Manichanh, C., Nielsen, T., Pons, N., Levenez, F., Yamada, T., Mende, D.R., Li, J., Xu, J., Li, S., Li, D., Cao, J., Wang, B., Liang, H., Zheng, H., Xie, Y., Tap, J., Lepage, P., Bertalan, M., Batto, J.M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H.B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H., Yu, C., Jian, M., Zhou, Y., Li, Y., Zhang, X., Qin, N., Yang, H., Wang, J., Brunak, S., Dore, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Bork, P. and Ehrlich, S.D., 2010. A human gut microbial gene catalogue established by metagenomic sequencing. Nature 464: 59-65.

Rayyis, S.F., Ambalavanan, N., Wright, L. and Carlo, W.A., 1999. Randomized trial of 'slow' versus 'fast' feed advancements on the incidence of necrotizing enterocolitis in very low birth weight infants. Journal of Pediatrics 134: 293-297.

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

Reichelt, K.L. and Knivsberg, A.M., 2009. The possibility and probability of a gut-to-brain connection in autism. Annals of Clinical Psychiatry 21: 205-211.

Rolfe, R.D., 1984. Role of volatile fatty acids in colonization resistance to *Clostridium difficile*. Infection and Immunity 45: 185-191.

Sandler, R.H., Finegold, S.M., Bolte, E.R., Buchanan, C.P., Maxwell, A.P., Vaisanen, M.L., Nelson, M.N. and Wexler, H.M., 2000. Short-term benefit from oral vancomycin treatment of regressive-onset autism. Journal of Child Neurology 15: 429-435.

Schwiertz, A., Gruhl, B., Lobnitz, M., Michel, P., Radke, M. and Blaut, M., 2003. Development of the intestinal bacterial composition in hospitalized preterm infants in comparison with breast-fed, full-term infants. Pediatric Research 54: 393-399.

Schwiertz, A., Taras, D., Schafer, K., Beijer, S., Bos, N.A., Donus, C. and Hardt, P.D., 2010. Microbiota and SCFA in lean and overweight healthy subjects. Obesity 18: 190-195.

Stecher, B. and Hardt, W.D., 2008. The role of microbiota in infectious disease. Trends in Microbiology 16: 107-114.

Stefanatos, G.A., 2008. Regression in autistic spectrum disorders. Neuropsychology Review 18: 305-319.

Stoll, B.J., Hansen, N.I., Bell, E.F., Shankaran, S., Laptook, A.R., Walsh, M.C., Hale, E.C., Newman, N.S., Schibler, K., Carlo, W.A., Kennedy, K.A., Poindexter, B.B., Finer, N.N., Ehrenkranz, R.A., Duara, S., Sanchez, P.J., O'Shea, T.M., Goldberg, R.N., Van Meurs, K.P., Faix, R.G., Phelps, D.L., Frantz III, I.D., Watterberg, K.L., Saha, S., Das, A. and Higgins, R.D., 2010. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. Pediatrics 126: 443-456.

Sturm, R., Staneck, J.L., Stauffer, L.R. and Neblett III, W.W., 1980. Neonatal necrotizing enterocolitis associated with penicillin-resistant, toxigenic *Clostridium butyricum*. Pediatrics 66: 928-931.

Suau, A., Bonnet, R., Sutren, M., Godon, J.J., Gibson, G.R., Collins, M.D. and Dore, J., 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Applied and Environmental Microbiology 65: 4799-4807.

Sudo, N., Chida, Y., Aiba, Y., Sonoda, J., Oyama, N., Yu, X.N., Kubo, C. and Koga, Y., 2004. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. Journal of Physiology 558: 263-275.

Talley, N.J., Abreu, M.T., Achkar, J.P., Bernstein, C.N., Dubinsky, M.C., Hanauer, S.B., Kane, S.V., Sandborn, W.J., Ullman, T.A. and Moayyedi, P., 2011. An evidence-based systematic review on medical therapies for inflammatory bowel disease. The American Journal of Gastroenterology 106 Suppl. 1: S2-25; quiz S26.

The, H.M.P.C., 2012. Structure, function and diversity of the healthy human microbiome. Nature 486: 207-214.

Thomas, R.H., Meeking, M.M., Mepham, J.R., Tichenoff, L., Possmayer, F., Liu, S. and MacFabe, D.F., 2012. The enteric bacterial metabolite propionic acid alters brain and plasma phospholipid molecular species: further development of a rodent model of autism spectrum disorders. Journal of Neuroinflammation 9: 153.

Thompson, A.L., 2012. Developmental origins of obesity: early feeding environments, infant growth, and the intestinal microbiome. American Journal of Human Biology 24: 350-360.

Tsai, H.H., Dwarakanath, A.D., Hart, C.A., Milton, J.D. and Rhodes, J.M., 1995. Increased faecal mucin sulphatase activity in ulcerative colitis: a potential target for treatment. Gut 36: 570-576.

Tsai, H.H., Sunderland, D., Gibson, G.R., Hart, C.A. and Rhodes, J.M., 1992. A novel mucin sulphatase from human faeces: its identification, purification and characterization. Clinical Science 82: 447-454.

Turnbaugh, P.J., Hamady, M., Yatsunenko, T., Cantarel, B.L., Duncan, A., Ley, R.E., Sogin, M.L., Jones, W.J., Roe, B.A., Affourtit, J.P., Egholm, M., Henrissat, B., Heath, A.C., Knight, R. and Gordon, J.I., 2009. A core gut microbiome in obese and lean twins. Nature 457: 480-484.

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

Turnbaugh, P.J., Ley, R.E., Mahowald, M.A., Magrini, V., Mardis, E.R. and Gordon, J.I., 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature 444: 1027-1031.

Tvede, M. and Rask-Madsen, J., 1989. Bacteriotherapy for chronic relapsing *Clostridium difficile* diarrhoea in six patients. The Lancet 333: 1156-1160.

Van den Abbeele, P., Van de Wiele, T., Verstraete, W. and Possemiers, S., 2011. The host selects mucosal and luminal associations of coevolved gut microorganisms: a novel concept. FEMS Microbiology Reviews 35: 681-704.

Vrieze, A., Van Nood, E., Holleman, F., Salojarvi, J., Kootte, R.S., Bartelsman, J.F., Dallinga-Thie, G.M., Ackermans, M.T., Serlie, M.J., Oozeer, R., Derrien, M., Druesne, A., Van Hylckama Vlieg, J.E., Bloks, V.W., Groen, A.K., Heilig, H.G., Zoetendal, E.G., Stroes, E.S., De Vos, W.M., Hoekstra, J.B. and Nieuwdorp, M., 2012. Transfer of intestinal microbiota from lean donors increases insulin sensitivity in subjects with metabolic syndrome. Gastroenterology, in press. DOI: http://dx.doi.org/10.1053/j.gastro.2012.06.031.

Wagner, R.D., 2008. Effects of microbiota on GI health: gnotobiotic research. Advances in Experimental Medicine and Biology 635: 41-56.

Wang, L., Christophersen, C.T., Sorich, M.J., Gerber, J.P., Angley, M.T. and Conlon, M.A., 2011. Low relative abundances of the mucolytic bacterium *Akkermansia muciniphila* and *Bifidobacterium* spp. in feces of children with autism. Applied and Environmental Microbiology 77: 6718-6721.

Wang, Y., Hoenig, J.D., Malin, K.J., Qamar, S., Petrof, E.O., Sun, J., Antonopoulos, D.A., Chang, E.B. and Claud, E.C., 2009. 16S rRNA gene-based analysis of fecal microbiota from preterm infants with and without necrotizing enterocolitis. ISME Journal 3: 944-954.

Williams, B.L., Hornig, M., Parekh, T. and Lipkin, W.I., 2012. Application of novel PCR-based methods for detection, quantitation, and phylogenetic characterization of *Sutterella* species in intestinal biopsy samples from children with autism and gastrointestinal disturbances. mBio 3: e00261-11.

Wright, D.P., Rosendale, D.I. and Robertson, A.M., 2000. *Prevotella* enzymes involved in mucin oligosaccharide degradation and evidence for a small operon of genes expressed during growth on mucin. FEMS Microbiology Letters 190: 73-79.

Wu, G.D., Chen, J., Hoffmann, C., Bittinger, K., Chen, Y.Y., Keilbaugh, S.A., Bewtra, M., Knights, D., Walters, W.A., Knight, R., Sinha, R., Gilroy, E., Gupta, K., Baldassano, R., Nessel, L., Li, H., Bushman, F.D. and Lewis, J.D., 2011. Linking long-term dietary patterns with gut microbial enterotypes. Science 334: 105-108.

Yap, I.K., Angley, M., Veselkov, K.A., Holmes, E., Lindon, J.C. and Nicholson, J.K., 2010. Urinary metabolic phenotyping differentiates children with autism from their unaffected siblings and age-matched controls. Journal of Proteome Research 9: 2996-3004.