ADDENDUM

Rebooting the microbiome

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ABSTRACT
Using a murine Salmonella model of colitis, we recently reported that mice receiving a community of defined gut microbiota (MET-1) lost less weight, had reduced systemic inflammation and splenic S. typhimurium infection, and decreased neutrophil infiltration in the cecum, compared to vehicle controls. In addition, animals receiving MET-1 exhibited preserved tight junction protein expression (Zonula occludens-1, claudin-1), suggesting important effects on barrier function. In this addendum, we describe additional in vitro experiments examining effects of MET-1, as well as in vivo experiments demonstrating that MET-1 is protective in a DSS model of colitis after administration of antibiotics. Placed in the context of our findings and those of others, we discuss differences in our findings between the Salmonella colitis and DSS colitis models, provide speculation as to which bacteria may be important in the protective effects of MET-1, and discuss potential implications for other GI diseases such as IBD.

INTRODUCTION
The number of bacteria present in the gut eclipses the total number of eukaryotic cells in the human body by many fold, and the vast majority of these live in the nutrient rich environment of the intestinal lumen where they can thrive as a community. Commensal bacteria are beneficial to gastrointestinal health, thus the manipulation of the gut microbiota to increase commensals or optimize microbial communities has been an area of active interest with regards to disease management. An extension of this concept has been the use of fecal microbial therapy (FMT) to treat infectious colitis from pathogens such as Clostridium difficile. FMT entails transplanting healthy donor fecal material containing commensal bacteria into the colon of a patient to reconstitute a healthy microbial community, and has proven very successful for the treatment of recurrent C. difficile infection (CDI). However, FMT has a poorly defined and non-reproducible composition and carries risks of unintended infectious organisms.

Work in our laboratories has attempted to create a defined Microbial Ecosystem Therapeutic (MET) with known organisms, in order to make a safe, reproducible therapy that simulates the beneficial effects of FMT. The mixture, designed to replace a dysfunctional, damaged ecosystem with a healthy ecosystem composed of ‘native’ intestinal bacteria, is known as “Repoopulate” or MET-1. The composition of MET-1 is shown in Table 1. It consists of 33 strains of commensal bacteria isolated from the stool of a healthy human donor, as previously described. Strains were selected based on antimicrobial resistance profiles, as well as on their ability to grow as a robust community in vitro in a continuous culture chemostat. Additional information on the functional and metabolic capabilities (or “metabolome”) of MET-1 has been described elsewhere. MET-1 has been used successfully in 2 patients to cure recurrent C. difficile infection that was refractory to standard antibiotic therapies.

In addition to protecting against C. difficile, there is increasing evidence that the gut microbiota play an important role in protecting the host against the deleterious effects of other intestinal pathogens, including...
Salmonella. Certain shifts in the gut microbiota (sometimes referred to as dysbiosis) contribute to this phenomenon. In animals, for example, treatment with a single dose of streptomycin will result in loss of colonization resistance, rendering mice more susceptible to developing Salmonella infection. The effect is similar regardless of whether streptomycin-sensitive or streptomycin-resistant strains of Salmonella are used, indicating that the antimicrobial susceptibility of the pathogen itself is of little importance. This observation has also been reported in humans, where exposure to antibiotics used to treat other infections increases the risk of developing Salmonella infection, with either Salmonella-resistant or Salmonella-sensitive strains.

We have demonstrated that MET-1 is protective against Salmonella enterica serovar Typhimurium (S. typhimurium) in a murine antibiotic model of Salmonella colitis. C57BL/6 mice, pretreated with oral streptomycin prior to receiving MET-1 or vehicle control, were then gavaged with S. typhimurium. The S. typhimurium-infected mice receiving MET-1 lost less weight, had reduced inflammatory cytokine levels in serum, and decreased neutrophil infiltration in the cecum. MET-1 also preserved tight junction ( TJ ) protein expression ( Zonula Occludens-1, claudin-1) in the cecum, and reduced metastatic spread of S. typhimurium infection to the spleen compared to vehicle controls. MET-1 did not appreciably kill Salmonella in vivo or decrease the intestinal burden of Salmonella in the intestine, suggesting that MET-1 confers protection through other mechanisms.

S. typhimurium and C. difficile are 2 different pathogens with quite differing mechanisms of pathogenicity. S. typhimurium relies heavily on host cell invasion and C. difficile uses primarily a toxin-mediated mechanism. Our discovery that MET-1 can protect against both of these organisms and both of these mechanisms of pathogenesis offers insight into core functions of healthy microbial communities. We will discuss our work using defined microbiota in different animal models of colitis, and describe additional data implicating enhancement of barrier function as an important mechanism of action. We are still exploring multiple possible mechanisms of action for MET-1, but in this addendum we will focus specifically on effects on barrier function and microbiota composition. We will also discuss potential implications for other GI diseases such as IBD.

Not just sitting on the fence: Gut microbiota protect by enhancing gut barrier integrity

One common unifying theme of colitis is inflammation leading to loss of barrier function. Intestinal barrier function is a key aspect of innate immunity, designed to physically limit bacteria to the intestinal lumen. Tight junctions between enterocytes at the apical aspect of the epithelium are comprised of...
transmembrane proteins that confer size and charge selectivity between the gut lumen and mucosa. In other words, tight junctions seal the space between adjacent epithelial cells. In addition to direct epithelial cell invasion, enteric bacteria pathogens can hijack host cell machinery by altering the structure and function of the TJ barrier. These effects may result from direct modification of TJ proteins, such as occludin, claudin and Zonula Occludens-1 (ZO-1), or by alteration of the perijunctional actomyosin ring. S. typhimurium specifically increases access to the basolateral compartment through disruption of the TJ complex and TJ protein localization, resulting in increased paracellular permeability and membrane "leakiness."18

Our Salmonella colitis study showed that compared to saline pretreatment control, pretreatment with MET-1 preserved localization of TJ proteins measured (ZO-1, claudin-1), after Salmonella infection. We additionally found decreased spread of Salmonella to distant organ sites such as the spleen in infected MET-1 animals compared to infected vehicle controls, confirming preservation of barrier function by MET-1. Interestingly, our findings showed that MET-1 had minimal effect on Salmonella viability, and no effect on direct Salmonella invasion of intestinal epithelial cells.16 It thus appears that the mechanism by which MET-1 attenuates systemic infection is by inhibiting the ability of S. typhimurium to gain access to the systemic circulation through the paracellular pathway; MET-1 preserves the integrity of the TJ complex.

To examine this in more detail, we used an in vitro model of altered barrier function in which T84 human intestinal epithelial cell monolayers were grown on transwell semi-permeable supports and co-cultured with either S. tmi/MET-1 or S. tmi/vehicle control in the presence of C. difficile toxin (which rapidly destroys the integrity of the actin cytoskeleton); transepithelial electrical resistance (TER) was used to measure barrier integrity (Fig. 1A). In mice, intestinal inflammation and neutrophil infiltration contribute to epithelial barrier function disruption; however for the in vitro experiment, C. difficile toxin was used as a tool to disrupt the epithelial cell barrier. Whereas monolayers treated with toxin alone or Salmonella + toxin had a decrease in TER, in the MET-1 + Salmonella + toxin samples the TER decreased only marginally, indicating preserved barrier function with MET-1 compared to saline control (Fig. 1B). In the toxin-treated group receiving MET-1, fewer Salmonella bacteria were recovered from the basolateral compartment of the transwell compared to the vehicle control group (Fig. 1C). This data further supports the proposed mechanism that MET-1 enhances barrier function to decrease Salmonella translocation via a paracellular route.16

Next, we considered whether claudin-2 would be affected by MET-1 pretreatment. Claudin-2 has been
recently described as a “leaky” intestinal TJ protein which plays an important role in water and solute regulation and is upregulated by *S. typhimurium* infection. MET-1/*S. tm* mice were observed to generally produce more formed stool than VC/*S. tm* mice. As shown in Figure 2A, claudin-2 expression in MET-1/*S. tm* mice was confined primarily to the bottom of the crypts and more closely resembled uninfected controls (VC mice and MET-1 mice). In contrast (and as has been previously reported), in VC/*S. tm* mice the expression of claudin-2 was more diffusely spread up the length of the crypts. This site specificity appears important as the overall amount of claudin-2 in the VC/*S. tm* mice and MET-1/*S. tm* mice was unchanged (Fig. 2B). Taken together, these data indicate that even during *Salmonella* infection MET-1 maintains the

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**Figure 2.** MET-1 pretreatment prevented the disruption of claudin-2 localization in the cecum caused by *S. typhimurium* infection. (A) Claudin-2 immunofluorescence staining (green) in ceca fixed in 10% formalin (400x magnification). Nucleic acid was stained using DAPI (blue). White arrows in picture show the green staining of claudin-2. Uninfected mice were pretreated with vehicle control (VC) or MET-1. Mice infected with *S. typhimurium* (*S. tm*) were also pretreated with either vehicle control (VC/*S. tm*) or MET-1 (MET-1/*S. tm*). VC/*S. tm* mice had increased claudin-2 epithelial cell immunofluorescence at the crypt base as well as in the mid and upper crypt zones and on the surface (compared to uninfected controls). This alteration in claudin-2 expression was attenuated in MET-1/*S. tm* mice. Scale bar represents 100 μm. (B) Expression of claudin-2 was measured by Western blot analysis of total cecal lysates (sample blot shown). Although panel (A) showed differences in localization, densitometric values calculated for the ratio of claudin-2 to β-actin showed no statistically significant difference between total expression of claudin-2 in MET-1/*S. tm* compared to VC/*S. tm* mice. Data were analyzed using a 1-way ANOVA with Bonferroni correction. *N* = 6 for each group. NS = not significant.
localization of claudin-2 at the bottom of the crypts, where it is normally expressed under conditions of intestinal homeostasis.

To further examine the effects of MET-1 on barrier function we utilized another murine model of colitis that disrupts barrier function, but does not involve infection with a specific pathogen. Dextran Sulfate Sodium (DSS) is a sulfated dextran molecule that can be dissolved in the drinking water of mice, leading to the development of colitis.\(^{21}\) DSS-induced colitis is one of the most commonly used mouse models of IBD and is thought to model human ulcerative colitis, although the mechanism of the disease is not well understood.\(^{22}\) It is thought that DSS can chemically compromise barrier function and allow for infiltration of microbes and luminal antigens to drive inflammation. The inability to maintain an intact barrier thus contributes to excessive translocation and sampling of intestinal microbes, resulting in a loss of homeostasis and resultant inflammation.

For this model, mice were pretreated 24 hours before MET-1 gavage with a combination of oral vancomycin and streptomycin. Both of these antibiotics are not absorbed into the circulation when taken by mouth, remaining in the gut lumen. Therefore, this combination of antibiotics has broad-spectrum effects on the gut microbiota, effectively creating a niche for other microbes to colonize. Following oral antibiotic treatment MET-1 was administered in a pretreatment regimen as described previously,\(^{16}\) and then mice were given 3% DSS. The hypothesized rationale for the efficacy of MET-1 pretreatment following oral antibiotics in the DSS model was that a decrease in indigenous microbes in the mouse colon would allow for stronger colonization of MET-1 following oral gavage, and that this microbial composition would be less conducive to the development of colitis following DSS administration. As predicted, MET-1 treated mice were less sick than control DSS mice (Fig. 3). Over the course of DSS exposure, mice that received MET-1 following antibiotics lost significantly less weight and displayed less histologic injury than controls. Effects on the immune response were further analyzed by measuring serum cytokine secretion levels. A decrease was noted in the average serum concentration for several inflammatory cytokines, including IL-1, IL-6, MCP-1, and IL-12 in antibiotic MET-1 DSS mice versus antibiotic saline DSS mice (Fig. 3B). The greatest cytokine decrease with DSS treatment occurred in chemokines important for acute phase inflammation, and myeloperoxidase (MPO) immunohistochemical staining also revealed reduced neutrophil infiltration into the colon of mice receiving MET-1 (data not shown). Both of these findings are similar to what was observed in the Salmonella colitis model with MET-1.\(^{16}\)

In contrast, there was a relative preservation of mucin-2 in the colons of antibiotic MET-1 DSS mice compared to the other treatment groups, an effect not observed with the Salmonella colitis model (Fig. 4). Mucin-2 is the primary constituent of the secreted mucous layers in the colon and it has a significant impact on intestinal homeostasis, acting as a physical barrier atop the epithelium. Loss of function of mucin-2 results in the development of spontaneous colitis\(^{23}\) and mucin-2 is reduced in the DSS colitis model.\(^{24,25}\) The ability of the microbiota to regulate the composition of the intestinal mucus has been demonstrated in vivo.\(^{25,26}\) In vitro, the probiotic Lactobacillus plantarum strain 299v was shown to upregulate mucin-2 mRNA in HT-29 cells.\(^{27}\) Upregulation of mucin-2 by other probiotic Lactobacilli has also been reported.\(^{28}\) In contrast, bacteria from Deferribacteraceae, and Verrucomicrobiaceae families, including an increase in the mucin-degrading genera Akkermansia, and Mucispirillum, are increased in DSS-treated mice.\(^{29}\) The latter microbes are known inhabitants of the gut that possess mucolytic ability. Taken together, these findings suggest that specific microbes may contribute to modulating the intestinal mucous barrier.

**Not all MET are created equal: Gut microbiota composition may be more important than we appreciate**

During DSS there are compositional changes in the microbiota, including increases in a number of different taxa. DSS results in an increase in the Proteobacteria family Enterobacteriaceae,\(^{29}\) which is represented in low quantities in MET-1. Interestingly, this family of microbes was seen to be disproportionately high in 2 patients with CDI, but was almost completely eradicated 6 months following treatment with MET-1, suggesting that MET-1 inhibits growth of this family.\(^{8}\)

Also in support of the role that microbes play in DSS-induced colitis is work demonstrating that certain probiotic organisms can curb disease progression. Oral pretreatment with a probiotic mixture containing
Lactobacillus spp. and four Bifidobacterium spp for one week prior to the induction of DSS-induced colitis was capable of significantly reducing weight loss and histological damage in mice. These changes were also correlated with restoration of certain microbes that were altered during colitis. The probiotic mixture VSL#3, comprised of 8 different strains belonging to the bacterial genera Bifidobacterium, Lactobacillus, and Streptococcus, was protective against DSS colitis by mechanisms involving limiting apoptosis in the colon and upregulation of tight junction proteins.

The IL-10 knockout (Il-10−/−) mouse model is another commonly used animal model for IBD. Changes to the microbiota that occur with progression of intestinal inflammation have been reported in this model. Increases in Proteobacteria and particularly in E. coli, as well as decreases in Firmicutes, microbial richness and overall diversity occurring over time have been described. These changes have also been reported in UC and CD patients. As with the DSS model, however, it can be difficult to discern whether these alterations of the enteric microbiota are a cause or effect of colonic inflammation.

Figure 3. MET-1 treatment following oral antibiotics prevented DSS-induced weight loss and histological damage. (A) Female, 7 week old C57BL/6 mice received MET-1 treatment following oral antibiotics (0.1 mg Vancomycin, 20 mg Streptomycin, or saline as vehicle control), prior to acute exposure to 3% DSS. Data analyzed by 2 way ANOVA with Bonferroni correction, (** p < 0.001). (B) Serum cytokine levels of IL-1β, IL-6, IL-12p70, and MCP-1 were significantly reduced in DSS mice pretreated with antibiotic + MET-1 compared to DSS mice pretreated with antibiotic + vehicle control. Serum cytokine levels were measured using a Bio-Plex Pro mouse cytokine magnetic bead kit. Both groups received antibiotics prior to DSS. Data were analyzed by Student T-test (** p < 0.05). (C) Representative photo of H&E staining of colons. DSS treatment resulted in a loss of mucosal architecture, and submucosal edema. MET-1 pretreatment following oral antibiotics protected against histological damage. N = 4 for each treatment group (N = 5 for DSS group). Scale bar represents 100 μm.
inflammation. In one Il-10⁻/⁻ study, the investigators concluded that the observed changes in the microbiota were secondary to the host immune response, although they noted that the dysbiotic changes observed in the composition of the microbiota may serve to further perpetuate colonic inflammation by contributing to, and perhaps even amplifying, the host inflammatory response.³⁴ Interestingly, FMT after ileocolic resection (ICR) in Il-10⁻/⁻ mice seems to protect against ileal inflammation post-operatively, but increase colonic inflammation.³⁶ The authors of this study concluded that increased inflammatory cytokines could act in concert with mucolytic pathobionts such as Klebsiella species, shown to be increased in the colonic samples, to culminate in the additive effect of increased bacterial translocation, a phenomenon previously reported to drive colitis and contribute to endotoxemia in this model.³⁶,³⁷

It is interesting to examine the composition of MET-1 in comparison to those taxa thought to be important in human IBD. The two major phyla in the healthy microbiota are consistently the Bacteroidetes and Firmicutes, highly represented in MET-1, whereas IBD is associated with an increase in Proteobacteria,³⁵ present in very low abundance in MET-1. The family Lachnospiraceae, which are well represented in MET-1, also appear to be significantly reduced in IBD microbiota. Clinically, we have already successfully used MET-1 as treatment for 2 patients with refractory, chronic CDI.⁸ Treatment with MET-1 stimulated expansion of Lachnospiraceae in both patients that were treated for CDI, suggesting the MET-1 community created a positive environment for these beneficial bacteria.⁸ More specifically, the organism Faecalibacterium prausnitzii, which we consider to be an integral member of MET-1, has been shown to be decreased in IBD patients suffering relapse, while its

Figure 4. MET-1 pretreatment following oral antibiotics attenuates DSS-mediated loss of Mucin-2 in the colon. (A) Representative photos of distal colons from antibiotic saline and antibiotic MET-1 pretreated mice, saline and MET-1 pretreated DSS mice and antibiotic saline, antibiotic MET-1 pretreated DSS mice. Mucin-2 appears green, nuclei stained using DAPI (blue). Scale bar represents 100 µm. Mucin-2 quantification data in (B) shows a statistically significant difference between Abx/DSS treatment and Abx/MET-1/DSS treatment, with more Muc-2 in the MET-1 treatment group, N = 4 mice per group. Data were analyzed by ANOVA with Bonferroni correction (**p < 0.0005).
abundance is thought to be an important factor in disease remission and is present to a high degree in MET-1. Conversely, taxa associated with the Proteobacteria family Enterobacteriaceae appear to be highly abundant in IBD but are represented by only 2 species in MET-1, and were significantly reduced in the 2 patients following MET-1 treatment. This is also true of *Streptococcus faecalis* micron strains of *Clostridia* (previously named *Desulfovibrio*), *Bacteroides* and *Enterococcus faecalis* which are in high abundance in IBD patients but low and not present in MET-1, respectively.

The fact that there are a number of taxa increased in DSS-induced colitis in mice, as well as in IBD in humans, suggests that changes to these taxa may contribute to development of disease. Furthermore, it appears as though changes to the microbiota of mice in this model occur at the onset of disease, hinting at causation. This suggests that microbial transplant could be efficacious in preventing or correcting dysbiosis. Many of these taxa occur in low amounts or not at all in the MET-1 mixture and abundance of some of these taxa was drastically inhibited by MET-1 in humans, comprising only a very small proportion of the community following treatment. The opposite is also true; certain taxa which are known to be decreased in IBD patients are very abundant in MET-1 and flourish following transplant into a host. These parallels provide a compelling link between microbial contribution to the inflammatory disease state and the potential efficacy of MET-1 treatment.

In addition to MET-1, other groups have similarly demonstrated that engineered communities can be used to treat human disease. For example, Tvede and Rask-Madsen created a mixture of 10 strains of gut-derived bacteria grown in pure culture and then administered these, resuspended in saline, by enema to treat *Clostridium difficile* infection. The mixture was comprised of *Clostridium innocuum*, *C. ramosum*, *C. bifidum*, *Bacteroides ovatus*, *B. vulgatus*, *B. thetaotaomicron*, *Enterococcus faecalis* (previously named *S. faecalis*), *Ruminococcus productus* (previously named *Peptostreptococcus productus*), and 2 strains of *E. coli*. Of the 6 recurrent CDI patients in the study, one received FMT, 4 received their defined mixture, and one patient who did not respond to FMT subsequently received the defined mixture, with good response. This study was performed in 1989 before the emergence of hypervirulent strains of *Clostridium difficile* but it was nevertheless effective in achieving cure, demonstrating the feasibility of a defined gut microbiota approach. Using an animal model, an even more narrowly defined mixture of 6 strains of bacteria (*Staphylococcus warneri*, *Enterococcus hirae*, *Lactobacillus reuteri*), and 3 novel species (*Anaerostipes sp.* and *Enterorhabdus sp.*) was shown to be effective in protecting mice against *Clostridium difficile* infection. Finally, as mentioned previously, defined probiotic mixtures such as VSL#3, which contains 8 different strains of bacteria, have shown some efficacy for other GI inflammatory conditions such as pouchitis.

It is important to remember, however, that the compositional structure of a given microbiota tells only half the story – the abundance profiles of certain taxa may not always be correlated to their significance within a community and the mere presence or absence of certain species is likely less important than the net functional output of the ecosystem members. In addition, secreted products from the bacteria likely contribute significantly to their protective effects. Bacteria are not static beings, but instead respond rapidly to their environment and secrete a plethora of chemical compounds, some of which are used as signaling molecules both among microbes, and between microbes and host. Production of small molecules, such as short chain fatty acids, by the gut microbiota is heavily influenced by ecosystem structure and available nutrients, and also by the inflammatory environment of the gut itself. The same concept applies to organisms that can be found in a “dysbiotic” community. For example, *E. faecalis* secretes gelatinase, a metalloprotease which both degrades the epithelial junctional protein E-cadherin and acts on PAR-2 to increase intestinal permeability, an effect recapitulated by supernatants from ulcerative colitis patient fecal samples. Therefore, as we begin to unravel the mechanisms of protection by therapeutic ecosystems such as MET-1, a thorough understanding of the microbial ecosystem metabolome, and the influence of this on the host, is warranted. Such a study will be complex since the same microbes may respond differently to exposure to different ecosystems.

**Future directions**

Dysbiosis may contribute to a variety of other gastrointestinal diseases and diseases in other organs. To better grasp the complexities of ecosystem function, future areas of study should focus on investigating effects of ecosystem members, on the bacterial metabolites...
released by these microbiota, and on the barrier function-enhancing capacity of defined microbiota, to determine which members of the community are critical for this important function. Further research in this area may lead to defined therapies that provide an optimally enhanced barrier function with the potential to protect against multiple other diseases, where barrier function disruption may play a critical role.

**Abbreviations and acronyms**

Abx  Antibiotics  
CDI  *Clostridium difficile* infection  
CFU  Colony Forming Unit  
DSS  Dextran Sulfate Sodium  
FMT  Fecal Microbial Therapy  
H&E  Hematoxylin and Eosin staining  
IBD  Inflammatory Bowel Disease  
ICR  Ileocolic resection  
LPS  Lipopolysaccharide  
LTA  Lipoteichoic Acid  
MET  Microbial Ecosystem Therapeutic  
MET-1  Microbial Ecosystem Therapeutic Mix ("RePOOPulate")  
*S. tm*  *Salmonella typhimurium*  
TLR  Toll-like receptor  
VC  Vehicle control

**Disclosure of potential conflicts of interest**

E.O.P and E.A-V. are co-founders of Nubiyota and have filed a patent for MET-1 through Parteq Innovations (Queen's University). The other authors have no conflict of interests to declare.

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