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RESEARCH PAPER/REPORT

Bacteroides ovatus ATCC 8483 monotherapy is superior to traditional fecal transplant and multi-strain bacteriotherapy in a murine colitis model

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ABSTRACT

Background and aims: Bacteriotherapy aimed at addressing dysbiosis may be therapeutic for Inflammatory Bowel Diseases (IBDs). We sought to determine if defined *Bacteroides*-based bacteriotherapy could be an effective and consistent alternative to fecal microbiota transplantation (FMT) in a murine model of IBD.

Methods: We induced experimental colitis in 8– 12-week-old C57BL/6 mice using 2–3% dextran sodium sulfate. Mice were simultaneously treated by oral gavage with a triple-*Bacteroides* cocktail, individual *Bacteroides* strains, FMT using stool from healthy donor mice, or their own stool as a control. Survival, weight loss and markers of inflammation (histology, serum amyloid A, cytokine production) were correlated to *16S rRNA* gene profiling of fecal and mucosal microbiomes.

Results: Triple-*Bacteroides* combination therapy was more protective against weight loss and mortality than traditional FMT therapy. *B. ovatus* ATCC8483 was more effective than any individual strain, or a combination of strains, in preventing weight loss, decreasing histological damage, dampening inflammatory response, and stimulating epithelial recovery. Irrespective of the treatment group, overall *Bacteroides* abundance associated with treatment success and decreased cytokine production while the presence of *Akkermansia* correlated with treatment failure. However, the therapeutic benefit associated with high *Bacteroides* abundance was negated in the presence of *Streptococcus*.

Conclusions: *Bacteroides ovatus* monotherapy was more consistent and effective than traditional FMT at ameliorating colitis and stimulating epithelial recovery in a murine model of IBD. Given the tolerability of *Bacteroides ovatus* ATCC 8483 in an active, on-going human study, this therapy may be repurposed for the management of IBD in a clinically expedient timeline.

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Introduction

The Inflammatory Bowel Diseases (IBDs), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic precancerous inflammatory conditions of the gastrointestinal tract, which affect over one million Americans.[1] Around 20% of all IBD presents in children and is associated with significant morbidity.[2] Pediatric IBD is frequently resistant to conventional medical therapy, with 20–25% of the patients requiring surgery within 5 years. These numbers increase to 35–40% of children requiring surgical intervention by 10 years follow-up.[3] Additionally, side effects from existing medications can be severe, including bone marrow suppression and malignancy.[4] Therefore, the development of novel therapeutic approaches towards IBD is an important medical need.

Disruption of the healthy gut microbial community (dysbiosis) has been implicated in the pathogenesis of many human diseases including *Clostridium difficile* infection (CDI),[5] irritable

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bowel syndrome (IBS),[6-10] and IBD.[11,12] Several studies have shown that patients with IBD exhibit decreased species richness and diversity as well as alterations in the abundance of several taxa compared to non-inflammatory controls.[13-15] Clinical observations regarding the efficacy of broad-spectrum oral antibiotic therapy for the treatment of IBD further indicate the involvement of the intestinal microbiome in these disorders.[16-18] Thus, it is hypothesized that therapies aimed at restoring microbial homeostasis can be beneficial treatment strategies for such disorders. Additionally, such therapies may offer the benefit of being safer than the immunosuppressive regimens commonly used to treat these conditions.

The most comprehensive approach to altering the enteric microbiota is via fecal microbiota transplantation (FMT), a procedure in which stool from a healthy donor is transferred into patients with the disease. Multiple studies have demonstrated that patients with active UC or CD report clinical improvement and even clinical remission in response to FMT.[19-24] However, others report conflicting results, with some studies showing that FMT does not confer any benefits to patients.[25,26] In fact, one study demonstrated that patients with IBD and CDI had improvement in their CDI, but lack of improvement in their IBD symptoms after FMT.[27] Recent meta-analyses of FMT as a therapy for IBD have demonstrated that FMT is well tolerated in IBD patients and that the pooled estimate of achieved clinical remission in UC patients is 24.1–40.5%,[28,29] as compared to 60.5% in CD patients.[28,30,31] However, the fecal microbiome is a highly complex and dynamic community that can vary within and between donors, leading to substantial variability in therapeutic efficacy and difficulties in deciphering the potentially key therapeutic attributes of FMT. Therefore, the identification of stable bacterial communities that carry the therapeutic effects of FMT could provide a more consistent and robust bacteriotherapy for IBD.

The Human Microbiome Project established that *Bacteroidetes*, of which *Bacteroides* is a prominent member, is one of the most dominant phyla in the healthy human intestine.[32] Patients with IBD have decreased levels of *Bacteroides* compared to healthy controls.[33,34] Furthermore, the level of *Bacteroides* is lower in IBD patients in the active phase of disease compared to those in remission.[34] These findings suggest that these *Bacteroides* species may be markers of human health, and their loss may be detrimental in the setting of IBD.

Tvede *et al.*[35] demonstrated the effectiveness of a 10-bacterial strain combination to successfully treat five patients with recurrent *Clostridium difficile* infection (rCDI), an infection also associated with dysbiosis.[36] The authors concluded that the three species in this combination contributing to remission belonged to the *Bacteroides* genus (*B. ovatus, B. thetaiotaomicron and B. vulgatus*). Importantly, the FDA has approved an IND application to test the safety and efficacy of this triple *Bacteroides* combination to treat humans with rCDI. Thus far, triple *Bacteroides* combination therapy has been well tolerated in adult recipients.

The negative association of these bacteria with IBD, the fact that IBD patients have a predisposition towards CDI[37] which may respond well to treatment with these bacteria, and the fact that these bacteria are well tolerated in adult patients, suggest that this combination of *Bacteroides* could also be effective for the treatment of IBD. In this work, we aimed to determine if defined bacteriotherapy (triple-*Bacteroides*, as well as the individual strains of *Bacteroides*) could be as effective as a complex bacteriotherapy (FMT) in a murine model of IBD. We then sought to elucidate the mechanisms behind inhibition of colitis by these various forms of bacteriotherapy.

Results

Triple-Bacteroides combination was protective against weight loss and mortality in DSS-colitis

In order to assess the efficacy of triple-*Bacteroides* as bacteriotherapy in a Dextran Sulfate Sodium (DSS) murine model of colitis, we assigned mice to 3 different groups, with 20 mice in each group: triple-*Bacteroides*, donor-recipient and self-recipient (Figure 1A). Mice received a bowel clean-out with Polyethylene glycol 3350 (PEG3350) for 48 h (as previously described by Ruggiero et al.[38]), followed by the administration of 3% DSS for 5 days concurrent to daily oral gavage of their

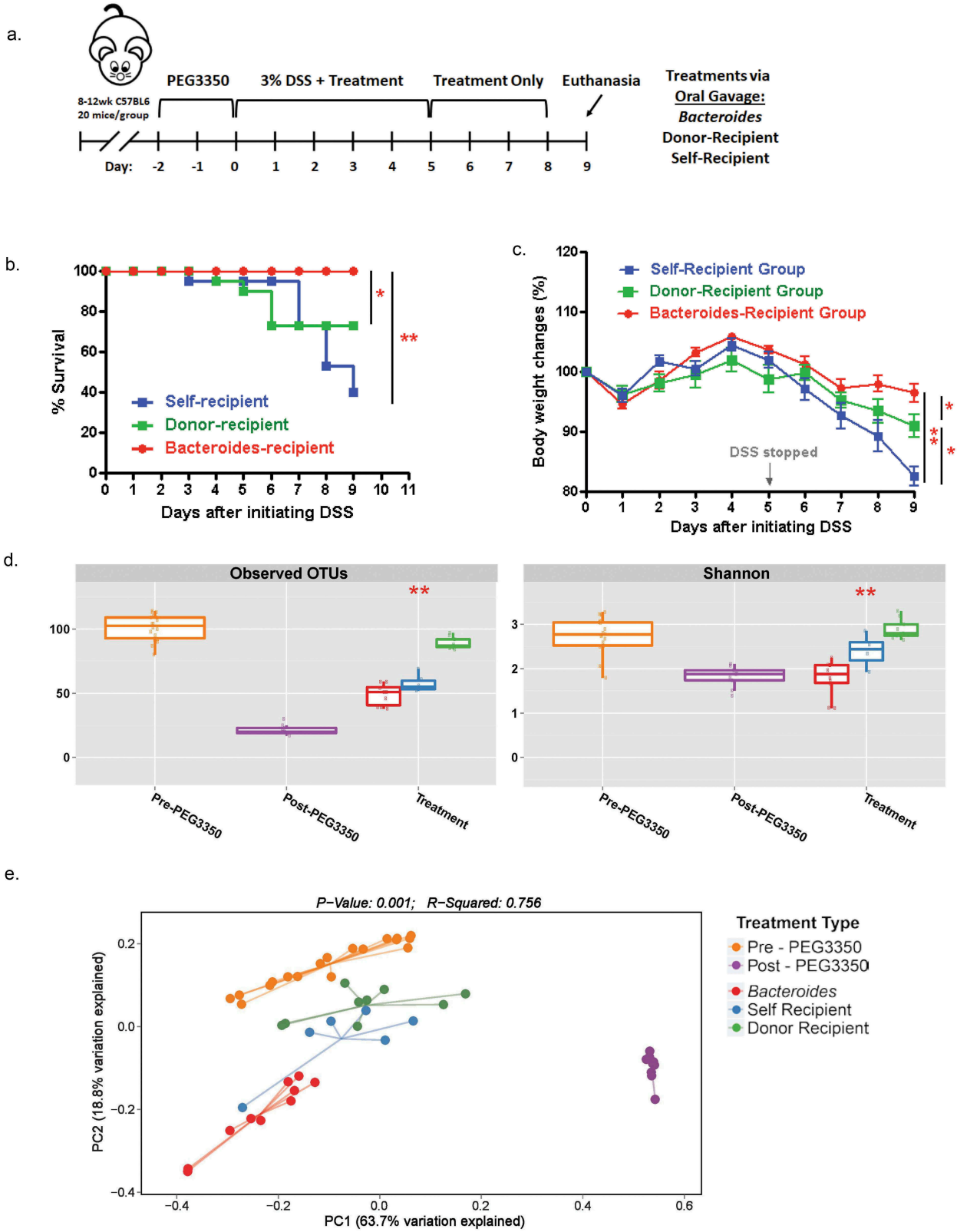

Figure 1. Triple-*Bacteroides* treatment protects against dextran sodium sulfate (DSS)-induced colitis. (A) Mice (n = 20 per group) pre-treated with PEG3350 for 48 h were then administered 3% DSS for 5 days concurrent to daily oral gavage (0.2 mL for each group) of *Bacteroides* combination (*B. ovatus, B. vulgatus* and *B. thetaiotaomicron* [*Bacteroides* Recipient]), fresh donor stool (donor recipient), or self-derived stool (self recipient). Following the cessation of DSS, mice were given normal drinking water and oral daily gavage of the respective treatments until termination. (B) Survival ($p < 0.05$) and (C) weight retention ($p < 0.001$ vs self, $p< 0.05$ vs donor) demonstrate the success of *Bacteroides* therapy relative to Donor and Self-recipient groups. (D) Restoration of microbial diversity and (E) microbiome structure (weighted UniFrac, stool only) was insufficient to confer beneficial effects on survival and weight retention. Pre-PEG3350 = healthy mice prior to bowel cleanout with PEG3350. Post-PEG3350 = all mice after 48 h of PEG3350 bowel clean out. Statistical analyses for A and B were performed using one-way ANOVA, followed by pairwise t-tests with adjusted p value thresholds (Tukey). For D and E, refer to statistics in the bioinformatics section.

respective treatments. Weight loss was monitored as a marker of colitis severity and *16S rRNA* gene profiling of fecal and mucosal microbiomes were conducted. Survival was found to be higher ($p < 0.05$) in the triple-*Bacteroides* group than in the self-recipient or donor-recipient groups (Figure 1B). Mice receiving triple-*Bacteroides* therapy lost 3.5% of their body weight, significantly less than the self-recipient (17.4%, $p < 0.001$) and donor-recipient (9.1%, $p = 0.03$) groups. Mice in the donor-recipient group also had less weight loss than the self-recipient group ($p = 0.01$, Figure 1C).

Restoration of microbial diversity and microbiome structure was insufficient to confer survival advantage during chemically induced colitis

As Expected, PEG 3350 cleanout reduced microbial richness and diversity. The 3-*Bacteroides* group exhibited the lowest number of observed OTUs and Shannon diversity in both fecal (Figure 1D) and mucosal samples (Supplementary Figure 2). After treatment, donor-recipient and self-recipient microbiomes more closely resembled pre-treatment (healthy) composition than the 3-*Bacteroides* group, with donor-recipient mice having restored richness (observed OTUs) and diversity (Shannon) to near-healthy levels. Donor-recipient mice regained much of their pre-PEG 3350 microbiome structure (Figure 1E) while the microbiome structure of *Bacteroides*-treated mice differed significantly (Weighted UniFrac, $p = 0.001$). Although the *Bacteroides*-recipient treatment group had the lowest level of diversity and highest dissimilarity to the pre-PEG3350 healthy microbiome, those conversely showed the most promising survival and weight outcomes.

B. ovatus ATCC 8483 was more effective than any individual strain, or combination of strains, in preventing weight loss associated with DSS-colitis

In order to determine the efficacy of the specific strains of *Bacteroides* to protect against colitis, we selectively tested each strain by randomly separating mice into five different groups (20 mice in each group): *B. ovatus, B. vulgatus, B. thetaiotaomicron*, 3-*Bacteroides* combination and self-recipient (Figure 2A). All mice receiving bacteriotherapy via individual *Bacteroides* strains or 3-*Bacteroides* combination survived (100%), as compared to only 42% of self-recipient ($p < 0.05$, Figure 2B). Mice receiving *B. ovatus* lost less weight ($p < 0.05$) compared to all other groups (Figure 2C). These findings encouraged us to proceed with and characterize the response to *B. ovatus* monotherapy.

B. ovatus mitigated weight loss, improved survival, and reduced inflammation in DSS-exposed mice

The high mortality rates of 3% DSS-exposed mice precluded unbiased mechanistic investigation. High mortality in the self-recipient groups reduced the final sample size in a treatment-specific manner at the termination of the experiment, thus preventing the inclusion of these samples in downstream analyses. For this reason, in all subsequent experiments, mice were administered 2% DSS (consistent with other published studies[39,40]) for 5 days concurrent with daily oral gavage (0.2 mL) of *B. ovatus* ATCC8483, fresh donor stool, or self-derived stool (Figure 3A). The experiment was terminated on day 6, before significant impact on survival, in an effort to capture all mice at the peak of colitis, but before the majority of the group would require euthanasia per protocol (Figure 3B). In order to more accurately simulate the non-related FMT donors that are typically used in human studies, we used three donor mice of the same age and genetic background, but from a separate litter housed in a different section of the facility. To determine disease severity, we measured several markers of inflammation-mediated damage including weight loss, changes in colon length/weight, histological damage scores, and SAA. Mice treated with *B. ovatus* had lost less weight ($p = 0.03$) than the self-recipient mice, in line with our previous findings (Figure 3C). Although colon length and colon weight were not significantly different between the treatment groups (Supplementary Figure 3), histological damage scores were significantly elevated in the self-recipient group relative to the *B. ovatus* group ($p < 0.001$, Figure 3D). Concordantly, mice in the *B. ovatus* group were observed to have less mucosal inflammation than the self-recipient

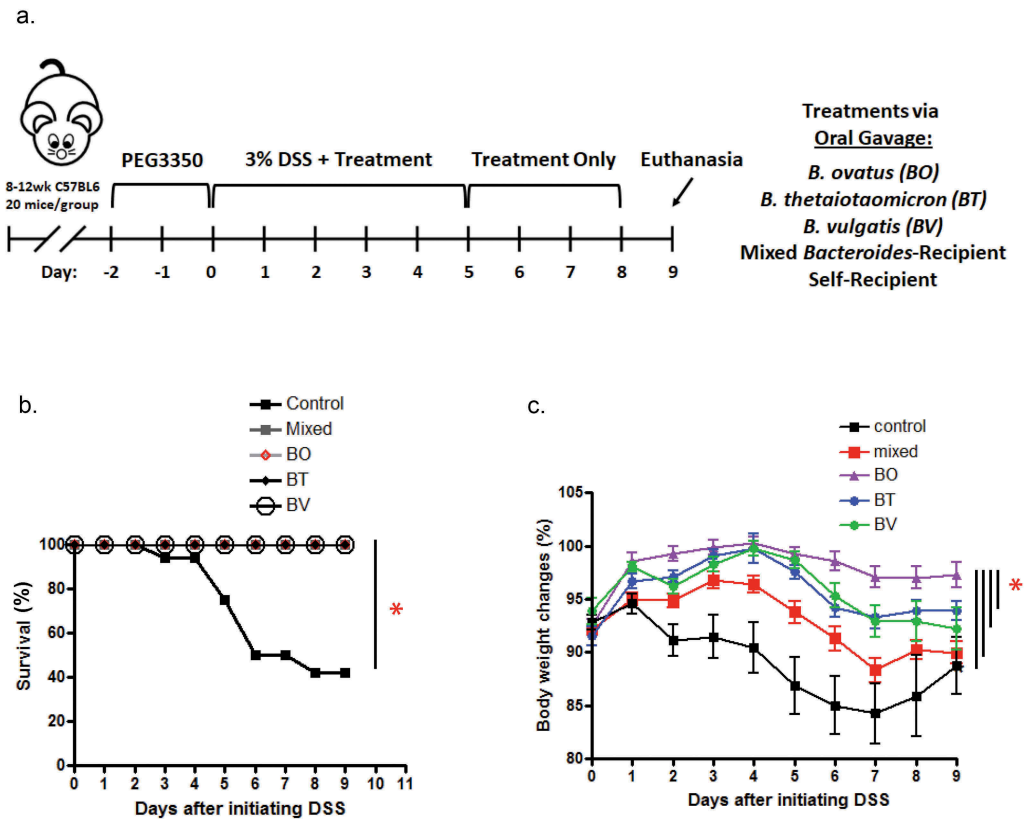

Figure 2. *B. ovatus ATCC8483* is the most effective in preventing weight loss associated with DSS-colitis. (A) Mice (n = 20 per group) pre-treated with PEG3350 for 48 h were then administered 3% DSS for 5 days concurrent to daily oral gavage (0.2 mL for each group) of each *Bacteroides* strain individually (*B. ovatus* [BO], *B. thetaiotaomicron* [BT], *B. vulgatus* [BV]), all three in combination (mixed), or self-derived stool (self-recipient). Following the cessation of DSS on day 5, mice were given normal drinking water and daily oral gavage until termination of the experiment on day 9. (B) Survival effect was not driven by any specific strain. (C) Weight retention of mice given any one strain was better than all three strains in combination, with *B. ovatus* as the most effective compared to all other groups ($p < 0.05$).

($p < 0.005$) and donor-recipient ($p = 0.016$) groups, as demonstrated by lower plasma SAA concentration (Figure 3E).

B. ovatus increased epithelial cell proliferation, goblet cell production, and crypt depth while ameliorating inflammatory response to DSS-colitis

Histologically, mice treated with *B. ovatus* had deeper, hyperplastic crypts ($p < 0.01$) compared to mice treated with FMT or autologous fecal transplant (Figure 3F). Also, mice treated with *B. ovatus* had increased epithelial cell proliferation based on IHC staining with Ki67 ($p = 0.02$) and increased goblet cell production based on Alcian blue staining ($p < 0.02$) (Figure 3(G, H)). This suggested that treatment with *B. ovatus* stimulated epithelial proliferation and mucin production during DSS exposure. Compared to the donor-recipient and *B. ovatus* groups, the self-recipient group had a markedly elevated inflammatory response to DSS, as demonstrated in Figure 3I. Comparatively, the donor-recipient group had a moderate inflammatory response, while *B. ovatus*-treated mice showed a stark lack of cytokine expression, including expression of IL-13, which is generally considered to be anti-inflammatory. These data suggest that *B. ovatus* treatment suppresses or prevents a cytokine-mediated response to DSS.

Bacteroides associated with weight retention, Akkermansia, and Streptococcus associated with weight loss in DSS colitis

Microbiome analyses were conducted on colonic mucosal and fecal microbiomes of the mice from this final experiment. Alpha diversity was similar

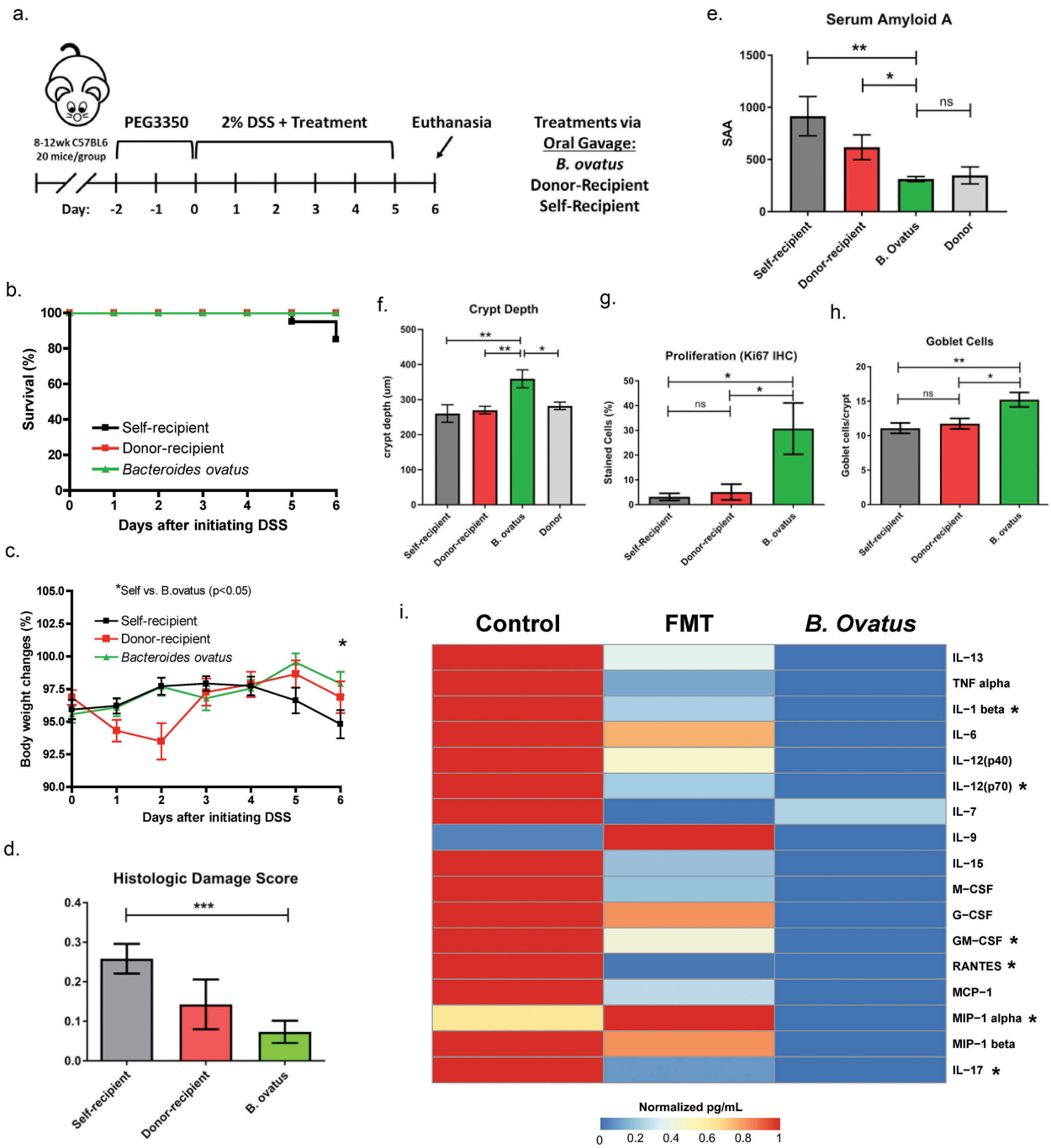

Figure 3. *Bacteroides ovatus* ATCC8483 is protective against DSS colitis. (A) Mice (n = 20 per group) pre-treated with PEG3350 for 48 hours were then administered 2% DSS for 5 days concurrent to daily oral gavage (0.2 mL) of *B. ovatus ATCC8483* (*Bacteroides ovatus*), fresh donor stool (donor-recipient), or self-derived stool (self-recipient). (B) Mice treated with *B. ovatus* retained significantly more (C) weight, had (D) decreased levels of histological damage, and experienced significantly less (E) mucosal inflammation as measured by Serum Amyloid A levels. Histologically, *B. ovatus* treatments resulted in epithelial proliferation as measured by (F) crypt depth/hyperplasia and (G) Ki67 stained cells. *B. ovatus* treatment also promoted differentiation into (H) goblet cells. (I) MagPix mouse cytokine array (serum) data suggests that *B. ovatus* treatment mutes inflammatory response to DSS, * = $p < 0.05$ via ANOVA. All other statistical analyses were performed using one-way ANOVA, followed by pairwise t-tests with adjusted p value thresholds (Tukey). Error bars = SEM.

between all three groups at the end of the experiment (Supplementary Figure 4a). Due to the introduction of a complex, unrelated donor, bacterial abundance data identified a large number of taxa that differed between treatment groups (data not shown). Because weight retention and SAA levels were our primary

metrics of treatment success, we grouped all mice based on treatment success, independent of treatment received. Treatment failure was defined as >4.5% weight loss while treatment success was defined as ≤4.5% weight loss. These values were modeled on the accepted definition of cancer cachexia being >5% weight loss but relaxed slightly to ensure that enough samples remained within each category to retain statistical power.[41] Weight category and SAA levels were inversely associated ($p = 0.03$, Supplementary Figure 4b), with the highest SAA belonging to mice with the lowest weights, regardless of treatment group. A subset of fecal and mucosal samples from each group (n = 10) underwent *16S rRNA* gene sequencing. The fecal microbiomes that showed higher relative abundance of *Bacteroides* and lower abundance of *Akkermansia* were associated with weight retention (Figure 4A). In mucosal samples, *Bacteroides* was the only discriminatory genus associated with treatment outcome, regardless of treatment received (Supplementary Figure 4c). LEfSe analysis, which considers effect size, was able to confirm these associations and detect additional low-abundance genera that were significantly different between the success and failure groups (Figure 4B). *Bacteroides, Anaeroplasma*, and *Lachnoclostridium* were associated with treatment success, whereas *Akkermansia* and *Streptococcus* were associated with treatment failure. The presence of *Streptococcus*, with or without the presence of *Bacteroides*, was indicative of treatment failure as assessed by increased weight loss (Figure 4C, F-Group 1). It is notable that, while the *B. ovatus* group had better weight retention than the control group, when considering all mice together, the *B. ovatus* group made up only 45% of the mice in the treatment success group (with 26% from the self-recipient group and 29% from the donor-recipient group), and 18% of the treatment failure group (with the self- and donor-recipient groups each contributing 41%). Therefore, it is likely that colonization with *Bacteroides* can incur a protective effect regardless of whether the *Bacteroides* is a defined probiotic, derived from a donor, or inherently present.

In order to verify that these correlations would be maintained, even in the absence of *B. ovatus* treatment, analyses were repeated excluding the *B. ovatus* treatment group (Supplementary Figure 5). When only the Self Recipient and Donor Recipient groups were analyzed, *Bacteroides* continued to associate with treatment success and *Akkermansia* remained associated with treatment failure (Supplementary Figure 5a). LEfSe analysis of the Self Recipient and Donor Recipient groups again confirmed these findings (Supplementary Figure 5b). Finally, dendrogram based on hierarchical clustering revealed that, even in the absence of *B. ovatus* specific therapy, higher abundance of *Bacteroides* (especially when combined with the presence of *Neisseria*) associated with treatment success, while a higher abundance of *Akkermansia* associated with treatment failure (Supplementary Figure 5c).

Correlations between the microbiome and serum cytokines support suppression of inflammatory response by Bacteroides

We hypothesized that the intermediate inflammatory response of the donor-recipient group (Figure 3I) was due to the variable levels of *Bacteroides* in that treatment group. We thus applied sPLS canonical correlation analysis to determine how the microbiome profiles correlate to the circulating blood cytokine profiles. Independent of treatment group, the high relative abundance of *Bacteroides* and *Anaeroplasma* (i.e., genera previously associated with treatment success) showed similar correlations with the intensity of tested cytokines. On the other hand, *Akkermansia* and *Streptococcus* (i.e., genera previously associated with treatment failure) also showed similar correlations with the cytokine intensity (Figure 5). Notably, *Akkermansia* and *Streptococcus* were associated with an upregulation of inflammatory cytokines, while the increased abundance of *Bacteroides* was associated with a downregulation of inflammatory cytokines. These results further support the role of *Bacteroides* in suppressing the inflammatory response to DSS-induced colitis. Furthermore, the dramatically different cytokine profiles of mice with a high abundance of *Bacteroides* and *Streptococcus* may contribute to the weight-based treatment outcome associations found in Figure 4 (the improved weight retention with high *Bacteroides* abundance was negated in the setting of high abundance of *Streptococcus)*. We repeated this analysis, excluding the *B. ovatus* treatment group, in order to verify that these cytokine to microbiome correlations would persist (Supplementary Figure 6).

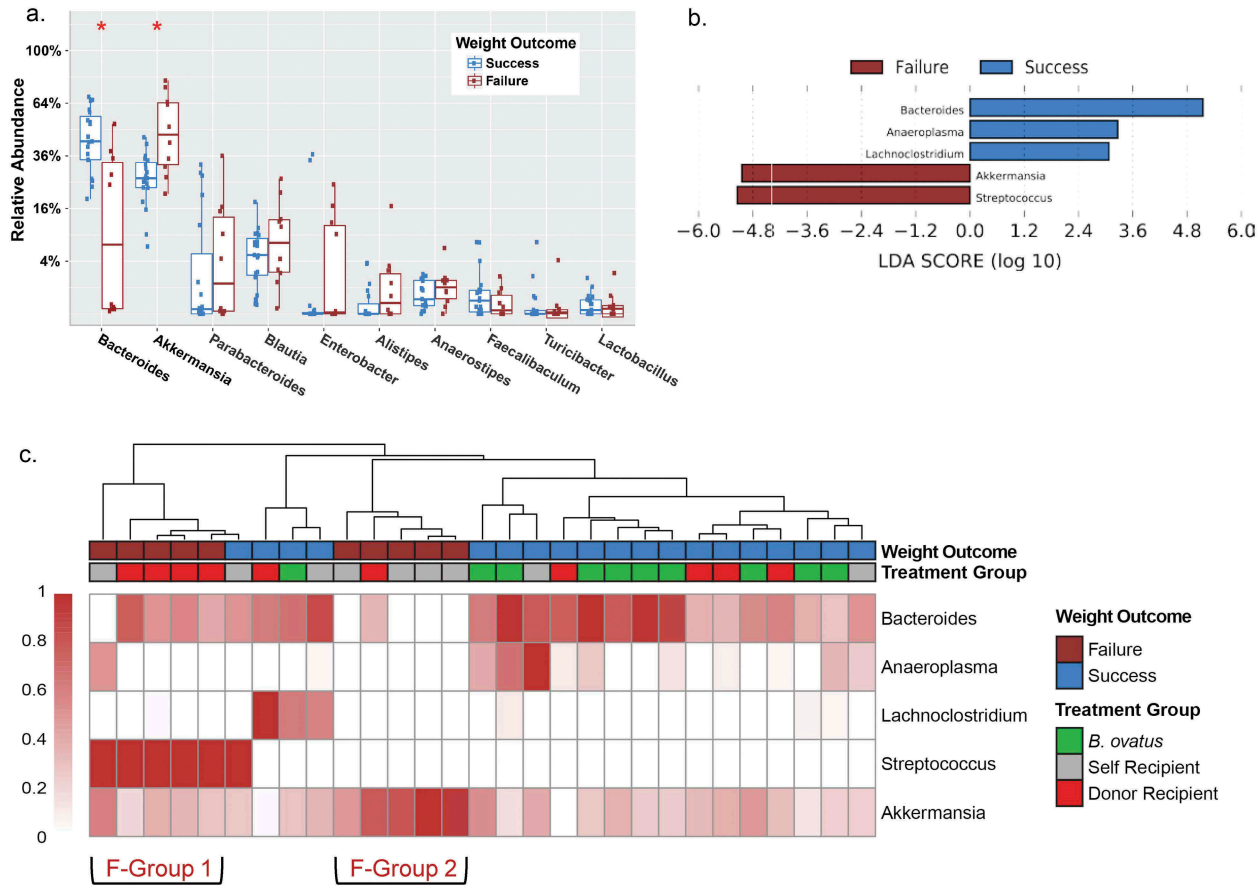

Figure 4. Fecal *Bacteroides* is associated with weight retention. Greater relative abundance of *Bacteroides* in the stool is associated with weight retention (i.e., treatment success) in DSS colitis, regardless of treatment group. (A) High relative abundance of *Bacteroides* is significantly associated with treatment success while high relative abundance of *Akkermansia* is associated with treatment failure. (B) LEfSe analysis (LDA score) corroborated these observations and also identified low abundance taxa associated with treatment outcomes. (C) Dendrogram based on hierarchical clustering reveals that greater abundance of *Streptococcus* led to worse outcomes (F-Group 1) and that mice low in *Bacterioides* but rich in *Akkermansia* (F-Group 2) were more likely to experience weight loss. Treatment groups included oral gavage of *B. ovatus* ATCC8483 (*B. ovatus*), self-derived stool (self recipient), or fresh donor stool (donor recipient). Weight outcome: failure = >4.5% weight loss, success = ≤4.5% weight loss.

The cytokine to microbiome analysis of Donor-Recipient and Self-Recipient mice suggested that a high abundance of *Bacteroides* and *Parabacteroides* was associated with reduced levels of circulating inflammatory cytokines. Alternatively, high relative abundance of *Akkermansia* correlated with increased levels of circulating inflammatory cytokines. These findings were very similar to the initial analysis that included all treatment groups, verifying the potentially significant role that *Bacteroides* and *Akkermansia* play in the inflammatory state during murine DSS colitis.

Discussion

The primary goal of this work was to determine if a defined bacteriotherapy (triple-*Bacteroides*, as well as the individual strains of *Bacteroides*) could be as effective as a complex bacteriotherapy (FMT) in a murine IBD model. We have shown that FMT, using stool from healthy donor mice, was effective at decreasing weight loss and increasing survival in DSS-induced murine colitis. This is in agreement with a recent study by Tian, *et al.*, which demonstrated the beneficial effect of FMT on DSS colitis in BALB/c mice, leading to increased survival and reduced disease activity index scores, colonic inflammation by histology, and inflammatory cytokine levels.[42] However, C57BL/6J mice in this study did not benefit from FMT. The investigators found that C57BL/6J and BALB/c mice responded differentially to the DSS, with C57BL/6J experiencing more significant weight loss and decreased survival compared to the BALB/c mice. This is likely a result of mice receiving 8 days of DSS followed by 8 days of FMT. Thus, by the

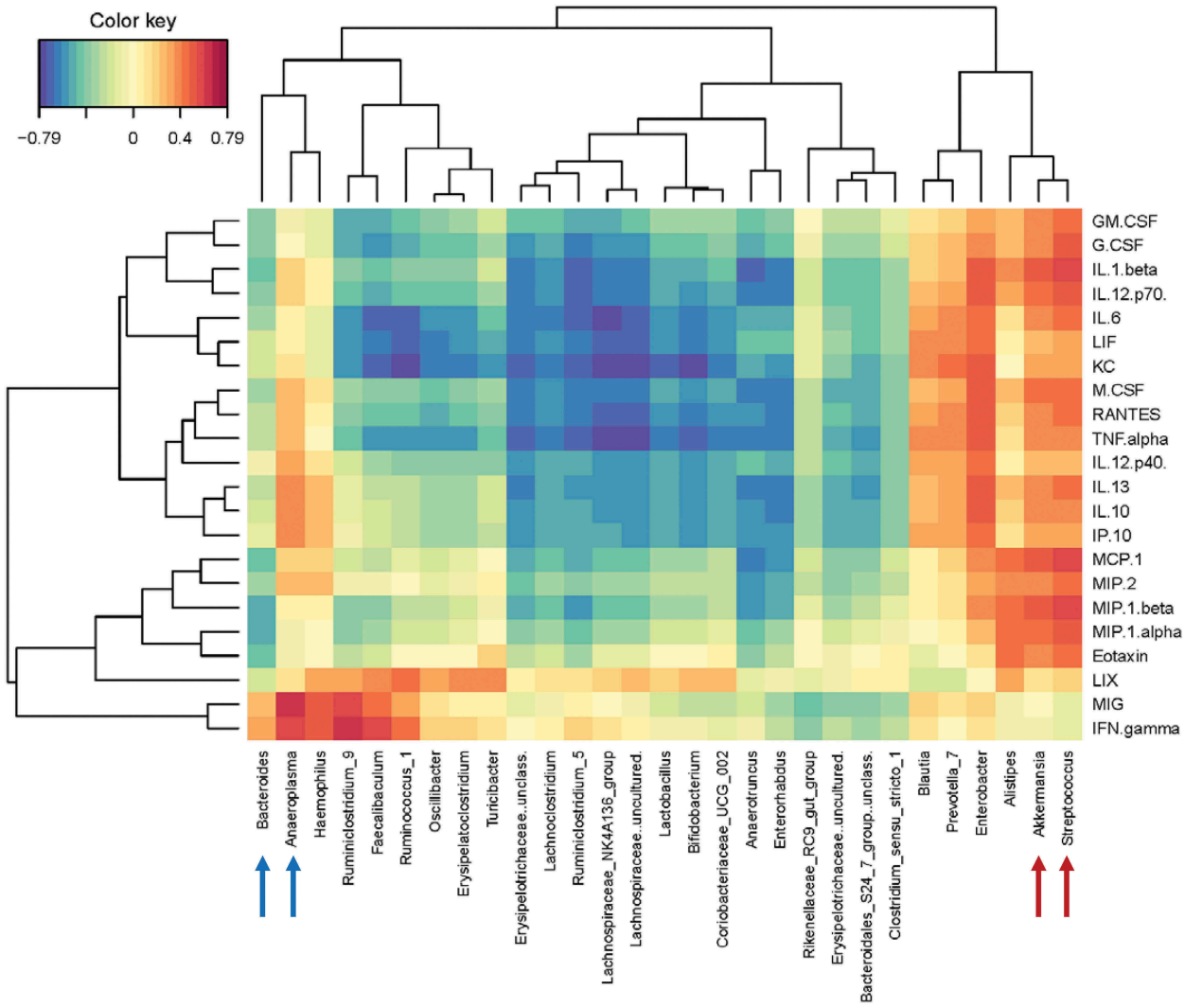

Figure 5. Fecal microbiome correlates with serum cytokine profile. The cytokine-to-microbiome analysis confirms that the cytokine profile of mice high in *Bacteroides* and *Anaeroplasma* is significantly different from mice high in *Akkermansia* and/or *Streptococcus*. sPLS canonical correlation analysis was used to determine how individual members of the microbiome correlate to circulating serum cytokines independent of treatment group. As illustrated in the dendrogram, mice with a greater relative abundance of *Bacteroides* and *Anaeroplasma* (blue arrows, genera previously associated with treatment success) exhibited a markedly different cytokine profile as compared to mice with greater abundance of *Akkermansia* and *Streptococcus* (red arrows, genera previously associated with treatment failure).

time the C57BL/6J mice received FMT, the colitis was already too severe to benefit from this treatment. In our study, FMT and DSS were given simultaneously, preventing the severe DSS-colitis phenotype. Notably, in the Tian, *et al.* study, mice treated with FMT had a significant increase in *Bacteroides* in their distal colon, which supports our finding that *Bacteroides* species contribute to the reduction of intestinal inflammation.

The presented data suggest that defined bacteriotherapy using strains of *Bacteroides* (specifically a triple-*Bacteroides* combination, as well as the individual strains of *Bacteroides)* is more effective at reducing colitis than a complex bacteriotherapy (FMT) in a murine model of IBD. Importantly, restoration of microbial diversity and microbiome structure by FMT was insufficient to confer a survival advantage over a triple-*Bacteroides* combination (*B. thetaiotaomicron, B. ovatus, B. vulgatus)*, which led to decreased richness and diversity, but improved overall survival, weight retention and decreased inflammation. This implies that *Bacteroides spp* are having a direct, protective effect against DSS-induced colitis.

There are limited studies examining the role of these strains of *Bacteroides* in the setting of colitis. *B. thetaiotaomicron* has demonstrated some potentially colitogenic properties,[43,44] while the impact of *B. vulgatus* on colitis is less clear, with some studies

suggesting a potential beneficial role against IBD, and others demonstrating a colitogenic quality.[33,45-47] Overall, *B. ovatus* appeared to be beneficial in the setting of colitis. A study by Saitoh *et al*. examining this question compared titers of serum antibody to commensal intestinal microbes in IBD patients and healthy controls, and found higher IgG and IgA titers to *B. ovatus* in patients compared to controls. The authors concluded that *B*. ovatus causes serum antibody responses in IBD patients, but stated that the role of this in the pathogenesis of these diseases is unclear.[48] Interestingly, monocolonization with *B. ovatus* in immunodeficient mice decreased mortality associated with DSS-induced colitis, demonstrating a potentially beneficial role.[49] Also, the presence of specific bacteria, including *B. ovatus*, could be used to distinguish healthy volunteers from patients with UC and IBS.[50] These findings imply that *B. ovatus* may be important in human health, and its loss may be harmful in the setting of these diseases. Furthermore, in our study, we found that the beneficial effects of *Bacteroides* may be negated by a low relative abundance of *Streptococcus*. Such interplay between bacterial genera has been emphasized in association with new-onset pediatric CD,[13] and this specific, potentially inhibitory relationship with respect to mammalian colitis warrants further investigation.

We have also found that increased abundance of *Bacteroides* in the stool was associated with weight retention during DSS colitis, regardless of treatment group. This may further emphasize the importance of this genus in acute colitis prevention. Additionally, a high abundance of *Akkermansia* was associated with weight loss and inflammation in our study. It is important to consider that these changes in the microbiome may be a consequence of the intestinal inflammatory state of the mice, as opposed to the cause of the increased or decreased inflammation. Alternatively, it is possible that these microbiome changes are critical in the development (or dampening) of intestinal inflammation. Oral administration of DSS disrupts the inner mucus layer by stripping the epithelium of sentinel goblet cells, allowing colonic bacteria to enter the crypts and stimulate the inflammasome via activation of toll-like receptor (TLR) ligands.[51] A recent study by Desai et al. demonstrated that mucinophilic bacteria, including *Akkermansia muciniphila*, could erode the colonic mucus layer, damage the epithelium, and increase host susceptibility to pathogens.[52] In our model, *Akkermansia* may be working in concert with DSS to enhance mucus layer disruption and stimulate the inflammasome. *Akkermansia muciniphila* has been shown to exacerbate the inflammation in gnotobiotic mice during co-infection with *Salmonella* Typhimurium.[53] Also, the presence of *Akkermansia muciniphila* has been associated with dramatic changes in the microbiome composition, most notable for a marked decrease in the *B. thetaiotaomicron* population.[53] This corroborates our finding that mice with the highest abundance of *Akkermansia* were less likely to have significant levels of *Bacteroides* (Figure 4C).

B. ovatus treatment was found to stimulate mucin production, increase proliferation/crypt depth, and decrease overall inflammatory activation in murine DSS-induced colitis. Increased proliferation of epithelial cells and elongated crypts have been used as markers of epithelial wound repair in murine models of colitis.[54] Similarly, enhanced mucus barrier formation has been shown to increase epithelial restitution in experimental models of colitis.[55,56] Consequently, *B. ovatus*-treated mice were better able to replenish the mucus layer as it is damaged by DSS, which may have prevented bacteria from infiltrating the crypts and activating the inflammasome. Furthermore, deeper crypts may boost this effect by increasing the distance bacteria must travel to stimulate pro-inflammatory TLR ligands. Our cytokine-to-microbiome analysis suggests that this effect is not limited to *B. ovatus*, but may be attributable to multiple *Bacteroides* species. Similarly, another strain of *Bacteroides, B. fragilis*, has also been shown to be able to dampen intestinal inflammation.[57,58]

We, therefore, theorize that *B. ovatus* acts directly on the gut epithelium to stimulate proliferation and differentiation into mucin-replenishing goblet cells, thereby limiting damage, microbial translocation, and immune activation during acute colitis. However, a further mechanistic investigation is necessary to address this hypothesis. We have also demonstrated that the cytokine profile of mice with high fecal *Bacteroides* is significantly different from mice with high fecal *Akkermansia* and *Streptococcus* during DSS colitis.

This further implies that *Bacteroides*, and specifically *B. ovatus*, may be able to dampen the inflammatory response in colitis by altering or preventing cytokine production, while *Akkermansia* and *Streptotoccus* exacerbate this response.

In conclusion, our data show that complex (FMT) and defined (*Bacteroides)* bacteriotherapy can be effective methods of improving outcomes in DSS colitis. *B. ovatus* monotherapy was the most effective at improving survival, decreasing weight loss and inflammation, and enhancing epithelial recovery during colitis. Restoration of healthy microbial diversity/structure was not responsible for this protective effect, but instead *B. ovatus* appeared to be able to suppress the DSS related inflammatory activation.

Despite these promising results and their clinical implications, we acknowledge several limitations of this study. The presented work does not address the question of bacterial colonization. Because these are human-derived *Bacteroides* strains, there is a strong possibility that they do not permanently colonize the murine gastrointestinal tract. However, in a clinical treatment scenario, lack of colonization may be favorable in case of adverse reaction to the bacterial strain, permitting clearance of *B. ovatus* without the use of an antibiotic. We also acknowledge that DSS-induced murine colitis is one of many available models of IBD. Repeating these experiments in another model, such as TNBS-induced colitis, or in a prospective human trial would elucidate the mechanism by which *B. ovatus* induces its beneficial effects and whether these responses are restricted to murine colitis. Further investigation will be necessary to address these questions.

It will be important for future studies to delineate the specific mechanism by which *B. ovatus* is able to provide its therapeutic benefit. While *Bacteroides* provided a protective effect during murine colitis, *Akkermansia* and *Streptococcus* were detrimental in this setting. This finding may be an important consideration when screening potential FMT donors, as the high abundance of these latter genera may prove harmful in the treatment of IBD and related disorders. Given the IND status of *B. ovatus* ATCC8483 and its tolerability in ongoing human investigations of rCDI, the results from this work may soon lead to a novel bacterial therapy to combat IBD.

Methods

Animals and tissue collection

C57BL/6J 8–10-week-old male mice (Jackson Laboratories, Bar Harbor, ME, USA) were utilized and maintained in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory animals. All the experiments were approved by the Institutional Review Board of the Baylor College of Medicine. At the time of euthanasia, a proportion of the mouse colons were placed on ice, transected longitudinally, cleansed from feces, and washed with normal saline. Colonic mucosa (excluding the cecum) were then collected with a microscope slide.[59] The mucosal scrapings were flash frozen on dry ice and stored at −80°C as previously described.[60] The remaining colons were longitudinally transected and processed, using the Swiss roll technique, for standard hematoxylin-eosin staining after fixation in 10% formaldehyde or Carnoy's fluid to preserve the mucus layer (placed in Carnoy's for 1–3 hours, stored in 80% ethanol).

Bowel cleanout with Polyethylene glycol 3350 (PEG 3350)

Based on the premise that bowel cleanout prior to FMT may increase the efficacy of FMT in humans with colitis, we applied this principal to our murine models of colitis. After 5 days of housing with daily cage mixing to allow for homogenization of the intestinal microbiome through the coprophagic behavior in mice, all animals received an osmotic laxative, PEG 3350 (31 mmol/L), in their drinking water for 48–-60 hours in an effort to decrease the bacterial load in their GI tract. This method of murine bowel cleanout was previously described by Ruggiero et al.[38]

Dextran Sulfate Sodium (DSS) exposure

Susceptibility to colitis was tested by administering 2% or 3% DSS (MW = 40,000–50,000, Affymetrix/USB, Thermo Fisher Scientific Chemicals, Inc., Ward Hill, MA, USA) in drinking water and provided ad libitum for 5–7 days. This molecular

weight of DSS has been shown to induce colonic inflammation in the previous work.[61] The animals were weighed daily, and colonic length measurements were performed at the end of the experiments following CO_2 asphyxiation or exsanguination by cardiac puncture. Weight loss (positively) and colonic length (negatively) correlated with histological severity of colitis in previous studies.[60,62] Therefore, we decided to follow weight loss and colon length as the primary clinical outcomes measuring colitis severity in our DSS experiments. Mice with more than 25% weight loss during DSS challenge were euthanized according to standard protocol at our institution. Additionally, colons were longitudinally transected and processed for histology and microbiome analysis.

Preparation of bacterial strains

The bacterial strains (*Bacteroides vulgatus* ATCC 8482, *Bacteroides thetaiotaomicron* ATCC29148 and *Bacteroides ovatus* ATCC8483) were grown on anaerobic reducible blood agar plates for 24–48 h, under anaerobic conditions. The bacteria were then harvested, and suspended in pre-warmed 35 saline to a concentration of 10^9 colony forming units (cfu)/ml. The bacteria in combination or as single strains were administered to the mice orally by gavage feeding (0.2 ml per mouse) within 2 h of harvest each day. OD600 measurements and viable counts were used to confirm the concentration of bacteria.

FMT and control delivery

To prepare FMT stool, freshly collected fecal samples from healthy donor mice were suspended in 2.5 mL of phosphate buffered saline (PBS) and administered (0.2 ml per mouse) immediately through oro-gastric gavage. Control mice received fresh fecal samples from their own stool, prepared daily, in the same manner. Given that mice are coprophagic, and therefore consume their own feces at baseline, oro-gavage of each mouse's own stool is an appropriate bacterial and procedural control for these experiments. The low volume of gavage (~5% of daily water consumption in mice) was considered to minimally influence the fluid balance of the animals.

16S rRNA gene sequencing and compositional analysis

Bacterial genomic DNA was extracted from using MOBIO PowerMag DNA Isolation Kit (MO BIO Laboratories). The bacterial 16S *rRNA* gene V4 region was amplified by PCR using barcoded Illumina adapter-containing primers 515F and 806R and sequenced with the 2 × 250 bp cartridges in the MiSeq platform (Illumina).[63] The read pairs were demultiplexed and reads were merged using USEARCH v7.0.1090.[64] Merging allowed zero mismatches and a minimum overlap of 50 bases, and merged reads were trimmed at the first base with a $Q \leq 5$. A quality filter was applied to the resulting merged reads and those containing above 0.5% expected errors were discarded. Sequences were stepwise clustered into Operational Taxonomic Units (OTUs) at a similarity cutoff value of 97% using the UPARSE algorithm.[65] Chimeras were removed using USEARCH v7.0.1090 and UCHIME. To determine taxonomies, OTUs were mapped to a version of the SILVA Database [66] containing only the 16S V4 region using USEARCH v7.0.1090.[64] Abundances were recovered by mapping the merged reads to the UPARSE OTUs. A custom script constructed a rarefied OTU table from the output files generated in the previous two steps for downstream analyses of alpha-diversity, beta-diversity (including UniFrac), and phylogenetic trends.

The datasets used and/or analyzed during the current study are available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA454325 (Accession number PRJNA454325).

Histology, microscopy, and image processing

Colons were longitudinally transected and processed, using the Swiss roll technique, for standard hematoxylin-eosin (H&E) staining after fixation in 10% formaldehyde. Histological severity of inflammation was determined by a blinded pathologist based upon a colitis scoring system previously reported by our group.[67] A portion of each tissue was fixed in Carnoy's fixative at room temperature for 3 h, stored in 80% ethanol, then embedded in paraffin wax. Sections (4 μm) were mounted on glass

slides, baked at 60⊠ for 1 h, then de-paraffinized with xylene and dehydrated in series from 100% to 50% ethanol. After standard H&E staining, histological severity of inflammation was similarly evaluated by a blinded pathologist. Slides were subsequently stained with Alcian blue to assess mucus layer thickness and integrity. Immunohistochemistry (IHC) was carried out according to standard procedures (Ki67 - Abcam, Cat# ab15580). Samples were imaged on a Nikon Eclipse E800 microscope using the Nikon NIS Elements Software (version 4.4) image capture software. Structurally intact crypts from a minimum of five fields (10 mice/group) were used for quantification of crypt depth, proliferation, and goblet cell number. The investigators were blinded to sample identity during both imaging and quantification. Quantification was performed using FIJI (ImageJ) software. Plots were generated using GraphPad Prism 7.

Immunological assays

Colitis severity was assessed by weight loss and serum amyloid protein A (SAA) concentrations. Animals were weighed daily throughout each experiment. SAA concentrations in plasma were measured using enzyme-linked immunosorbent assay (ELISA) kits from Alpco (Salem, NH), according to the manufacturer's protocol instructions.

The concentrations of murine interferon (IFN)-γ, interleukin (IL)-1α, IL-1β, IL-2, Il3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, keratinocyte chemoattractant (KC), tumor necrosis factor (TNF)-α, granulocyte colony-stimulating factor (G-CSF), Eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), LIX, IP-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, macrophage-colony stimulating factor (M-CSF), MIP-2, monokine induced by IFN-γ (MIG), RANTES, and vascular endothelial growth factor (VEGF) in the plasma were measured using cytokine multiplex kits (Lot# 2825623, Millipore, Billerica, MA). Quantification of cytokines was performed using the Luminex system (Austin, TX) according to the manufacturer's instructions. Briefly, plasma samples collected from each mouse were thawed completely and diluted 1:2 with Assay Buffer provided in the kits. The assays were performed in duplicate blindly (S.V.). The reports automatically generated by MILLIPLEX Analyst software version 5.1 (Millipore) were reviewed, and only cytokines that were greater than the lower limit of detection and below the saturation value were considered. To removed spurious associations, cytokine array data included in the generated heatmap (Figure 2I) met a significance threshold of $p < 0.2$ as calculated by an unadjusted one-way ANOVA and was normalized by row/cytokine. Heatmaps were generated in R using the pheatmap package.

Statistical and bioinformatic analyses

Analysis and visualization of microbiome communities were conducted, in part, in ATIMA, an R[68] software suite developed in-house at Baylor College of Medicine combining publicly available packages[69,70] and purpose written code to import sample data and identify trends in taxa abundance, alpha-diversity, and beta-diversity with sample metadata (Supplementary File 1). The significance of categorical variables was determined using the non-parametric Mann–Whitney test[71] for two category comparisons or the Kruskal-Wallis test[72] when comparing three or more categories. Correlation between two continuous variables was determined with linear regression models, where p-values indicate the probability that the slope of the regression line is zero. Principal coordinate plots employed the Monte Carlo permutation test[73] to estimate p-values. All p-values were adjusted for multiple comparisons with the FDR algorithm.[74] MixOmics was implemented to determine correlations between the microbiome and cytokine profiles based on sparse partial least squares regression (sPLS) and was performed in a canonical mode with LASSO penalization.[75]

Linear discriminant analysis (LDA) effect size (LEfSe) was performed using the Galaxy interface.[76] LEfSe uses the non-parametric factorial Kruskal-Wallis (KW) sum-rank test to detect features with significant differential abundance between success or failure groups. LEfSe further uses Linear Discriminant Analysis (LDA) to estimate the effect size of each differentially abundant feature. An LDA cut-off of 2.0 and significance value (P) of 0.05 were applied.

Ethics approval

All mice were utilized and maintained in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory animals. All the experiments were approved by the Institutional Review Board of the Baylor College of Medicine (protocol AN-5351).

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Disclosure of Potential Conflicts of Interest

J.F.P. is Founder and CSO of Diversigen, Inc, and a consultant for 4D Pharma PLC and DaVoltera. All other authors report no conflicts of interest.

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