Limitation of sulfur-containing amino acid availability by specific bacterial populations during enhanced colitis in IBD mouse model

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Abstract

The microbiota has profound influence on the host through interactions with the immune system early in host development. These interactions are crucial in understanding complex autoimmune diseases, such as Inflammatory Bowel Disease (IBD). Here, we examined how specific groups of microbes in dysbiotic mice impacted outcomes of colitis, host immune response, gene expression, and microbial functional changes. Vertically transmitted dysbiotic pups received fecal microbiota transplantation (FMT) from control mice, at 2, 3, and 8 weeks after birth. After 23 weeks, remaining mice were supplemented with 2.5% dextran sulfate sodium to induce colitis. Colon histology revealed mice receiving FMT displayed less colon inflammation than mice with no gavage. We assembled 190 non-redundant Metagenome-Assembled Genomes (MAGs) from pup fecal content and highlighted microbial community differences in FMT mice compared to dysbiotic groups. *Enterococcus* sp. and Enterobacteriaceae members were highly detected in dysbiotic mice only. These MAGs shared a large number of antimicrobial resistance genes as well as several virulence factors including a T6SS along with multiple inflammation inducing metabolic pathways. The Enterobacteriaceae sp. displayed the ability to uptake and utilize cysteine and taurine for sulfur metabolism, in addition to utilizing the host immune response production of nitrate and nitrite as energy sources. Here, we find that potentially pathogenic microbes in the dysbiotic gut have the metabolic capability to compete with the host for sulfur-containing amino acids as well as host derived nitrate and nitrite to further inflame the gut, and worsening IBD symptoms.

Keywords

Fecal microbiota transplantation, inflammatory bowel disease, microbial-host interactions, amino acid uptake, RNA sequencing, shotgun metagenomics

Introduction

Inflammatory bowel diseases (IBDs) are chronic autoimmune disorders with a wide range of outcomes and an unresolved etiology. Incidences of IBD are increasing globally, with 6 million cases, and an increasing number of diagnoses reported within the last 3 decades (1). The tri-factor of genetic, environmental, and microbial components are suspected in the development of these complex disorders (2–4). In addition to these factors, early life exposure of the gut to antimicrobials are reported to be correlated with development of IBD disorders (2, 5). Early exposure to antimicrobials leads to a dysbiotic state of the gut, where the resident microbiota is thrown into imbalance, which can lead to further inflammation (6). Microbial prevention methods such as fecal microbiota transplantation (FMT) are actively being investigated as a therapeutic for IBD or other related diseases like ulcerative colitis and Crohn's disease (7, 8). However, the outcomes from these trials have been inconsistent, leading to numerous researches racing to understand the microbial functions in these FMT, to provide insights into conflicting results from the transplantation (9–11).

Specific microbes have been hypothesized to play a role in the development or aggravation of IBD outcomes (12–14). Emergence of adherent invasive *Escherichia coli* (AIEC) and *Enterococcus faecalis* has been observed in IBD and Crohn's disease (CD) patients and harbor virulence factors found at higher rates than in healthy patients (13–15). However, we still have limited understanding of the interactions between these emerging harmful bacteria and the host during the onset of IBD and related inflammatory disorders. Furthermore, besides the identities of the relevant microbes impacting the outcomes of IBD, functions of these microbes before and during disease and how they interact with the host are crucial in understanding this complex disease dynamic. For example, *Akkermansia muciniphila* and butyrate producing *Clostridia* have been shown to improve gut physiology and even alleviate symptoms of IBD and inflammation (16–18). On the other hand, AIEC has been proposed as a possible cause for the beginning of the disease in IBD patients, and studies have also found an increased number of virulent *E. coli* strains isolated from IBD patients. Some studies suggest that the accumulation of AIEC and other *E. coli* strains in the gut is a consequence of inflammation, and several other studies revealed AIEC infection could result in changes in the gut microbiota. Currently, it remains unclear the underpinning mechanism of bacterial populations in the dysbiotic associated gut inflammation.

In IBD patients, the chronically inflamed intestine and colon are subjected to considerable oxidative stress. Amino acid metabolism plays an important role in the nutrition and physiology of the host and may influence the progress of IBD. Studies demonstrated that amino acids are used as building blocks for mucosal wound healing and energy substrates of enterocytes. These amino acids may act to reduce inflammation, oxidative stress, and the levels of proinflammatory cytokines. Although experimental studies investigating the efficacy of amino acids are promising, some clinical studies with supplementation in IBD patients are disappointing. Conflicting results from studies warrant a deeper understanding of the role of amino acid metabolism and the underlying mechanisms of IBD.

Here, we demonstrate that FMT early in development of vertically transmitted dysbiotic IL-10 KO mice had protective effects from the development of IBD later in life. In this study, we evaluated the effects of several compounding factors of IBD. To model an environmental impact contributing to IBD, we utilized vertical transmission of dysbiosis by treatment of dams with a broad-spectrum antibiotic leading to dysbiotic pups and investigated the protective effects of reintroducing a community of microbes to the intestinal tract, via FMT at two weeks after birth. Our results indicated that FMT enhances microbial diversity in the colon long-term, lowering colon inflammation, and interacts with the host immune system. Metagenomics provides us with a glimpse into the near reconstitution of a microbiota in mice receiving FMT, and the potential microbial functions to compare FMT and dysbiotic mice, elucidating important bacteria and their functions in both protected mice and mice experiencing colitis.

Results

Fecal content from pups were collected at the final week (week 24) of the study. Genomic DNA was extracted, and shotgun sequencing was conducted. Figure 1 shows the experimental setup and treatment protocol of the study. Briefly, mouse pups displayed vertically transmitted dysbiosis, except for the control group, and received either FMT, saline gavage, or no gavage during and after development. We employed genome-resolved metagenomics for an in-depth characterization of the gut microbiota and combined with host response markers - colon RNA sequencing, serum immune markers and histology to provide insights into the pathogenesis of the microbiota.

FMT resulted in reduced colon inflammation and changes in systemic serum markers

We investigated the host response to FMT to gain a larger perspective of the impacts later in life from re-introducing microbes during development of the neonates. We noticed that mice that received FMT displayed less colon surface damage, fewer infiltrating lymphocytes, and significantly lower inflammation scores than dysbiotic mice that did not receive FMT (p-value: <0.02). Mice that received the saline gavage or no gavage displayed the highest histopathological score with evidence of surface damage, lymphocyte infiltration, and loss of goblet cells (p-value: <0.029) (Figure 2A). Select systemic serum markers indicative of broad-scale inflammation were measured in IL-10 KO serum. The cytokine panel revealed low level trends for the control mice compared to other groups (Figure 2B). Notably, FMT mice resulted in significantly higher levels than the control mice in G-CSF (p-value 0.0011), IL-12p70 (p-value 0.044), IL-17 (p-value 0.025) TNFa (p-value 0.0403), and IL-6 (p-value 0.0028). One cytokine, IL-2, displayed lower levels in dysbiotic mice compared to FMT, however was not significantly different than control mice (p-value < 0.049). With few significant increases in common IBD inflammation indicators like IL-1beta and IL-17, we see little evidence of a systemic, uncontrolled, immune response, regardless of treatment group. In summary, IL-10 KO mice receiving FMT displayed less colon inflammation indicating a successful FMT with long term inflammation relief and cytokine modulation.

Upregulation of several IBD and cancer related genes in dysbiotic mice highlight importance of microbial biodiversity

To further investigate host response to long term dysbiosis, colon samples (n=3) from each treatment group underwent RNA sequencing. First, a Gene Ontology (GO) Analysis was performed to gain insight into broad gene categories that are strongly upregulated or downregulated by treatment group comparisons. Six pairwise comparisons revealed gene regulation changes between treatment groups (Supplementary Figure). Comparisons between control mice and FMT indicated few gene regulation differences, with only 6 gene categories significantly different, (3 classes upregulated, 3 downregulated). More differences were seen with comparisons between FMT and the saline gavage (35 upregulated, 32 downregulated; 67 total), as well as FMT against no gavage (84 upregulated, 45 downregulated; 129 total). Similarly, comparisons between control mice versus the saline gavage (20 upregulated, 34 downregulated; 54 total), and the no gavage groups (59 upregulated, 29 down; 88 total) also

resulted in several categorical differences. The highest number of significant gene categories was comparing the saline gavage to no gavage groups (53 upregulated, 100 downregulated, 153 total). Significant gene families between the dysbiotic groups and FMT mice include multiple inflammatory cytokine/chemokine activity, sulfur dioxygenase/ sulfur transport activity, cysteine receptor binding, and nitric oxide synthase binding. Gene family differences between control mice and dysbiotic groups indicated nearly identical trends. Finally, differences between FMT and control groups were fewer, with gap junction channel activity, cytokine/chemokine activity, specifically IL-17 receptor activity, and sulfur compound binding indicating the FMT stabilizes host gene expression. These gene families were examined because of the implications in the development of IBD in human patients. As discussed previously, cytokine and chemokine modulate kickstart inflammation during an immune response, with IFN gamma, IL-6, and IL-17 acting as early indicators of IBD (19). Sulfur dioxygenase activity is a precursor step in the production of cysteine, along with the transporter genes to gain access to more sulfur ions to construct cysteine (20). In IBD patients, low cysteine and taurine levels have been observed with pathogenic enterics like *Escherichia coli* benefiting from sulfur metabolism (21, 22), indicating from our data that dysbiotic mice may have lower cysteine levels contributing to the upregulation of cysteine biosynthesis gene families. Nitric oxide is also a crucial part of the host immune response to potential pathogens, nitric oxide is generally toxic to gut microbes (23), with this gene family being significant in dysbiotic mice, suggesting the presence of a pathogenic immune response and is another potential biomarker indicative of IBD (24).

Transference of microbial community via FMT to inherently dysbiotic IL-10 pups

Here, we examined the efficacy of re-introducing a microbial community via FMT early in life on the gut microbial composition post-development. We reconstructed metagenome-assembled genomes (MAGs) from the co-assembled treatment group sample metagenomes by grouping contigs into putative bins based on sequence composition and differential coverage signal (25, 26). We manually refined bins that satisfied >70% complete and <10% redundancy as predicted by bacterial single-copy core genes into MAGs. We resolved a total of 677 MAGs, and then removed redundancy by selecting a single representative for each set of genomes, resulting in 190 non-redundant MAGs (Supplementary Table S2) from the 4 experimental groups. We observed that there was a large representation of Firmicutes (n=178) with Proteobacteria (n=6), Bacteroidota (n=1), Actinobacteria (n=3) and Verrucomicrobiota (n=2) having lower detection. At the class level, Clostridia (n=158) was the most dominant, followed by Bacilli (n=17),

Gammaproteobacteria (n=3), Coriobacteriia (n=3), Negativicutes (n=1), Dehalobacteriia (n=1), Bacteroidia (n=1), Verrucomicrobia (n=1) and five remained unidentified (Figure 4A).

Clustering pattern of the samples based on the detection of the MAGs showed that gut microbiota of the samples that received FMT resembled more of the mice that were not dysbiotic. On the other hand, dysbiotic mice that either received gavage of PBS or no gavage were distinctly different from the non-dysbiotic and FMT mice (Figure 4B, Supplementary Table S3). We used the MAG's detection values to calculate the Bray-Curtis dissimilarity and non-metric multidimensional scaling (nMDS) and performed permutational multivariate analysis of variance (PERMANOVA) to show that there was a significant clustering of control/no-CPZ and CPZ/FMT group (Figure 2B). We also observed a wider clustering pattern in the CPZ/no-gavage and CPZ/gavage-with-saline groups. The differences among the treatment groups were mainly attributed to the absence and presence of the families Enterococcaceae and Enterobacteriaceae which exhibited largest differences in MAGs' detection (Supplementary Table S3).

We used the Subsystem-based approach and observed that the clustering of the samples corroborated with that of their detection in the samples (Figure 4C). PCoA plots that were based on Bray-Curtis dissimilarity and PERMANOVA clustering analysis revealed significant, distinct clustering of the different treatment groups. Of the 190 non-redundant MAGs, we noticed 7 MAGs that were of interest to our study - MAGs 001 and 002 (hereafter referred as MAGs-*Akkermansia*), MAGs 165 and 168 (hereafter referred as MAGs-*Enterococcus*), MAGs 166, 167, and 169 (hereafter referred as MAGs-Enterobacteriaceae). Detections of both *Enterococcus* MAGs and Enterobacteriaceae by treatment group indicate a lower presence in control and mice that receive FMT, with significantly higher detections in the dysbiotic mice with either saline or no gavage.

Linking host and microbe attributes to worsened outcomes in IBD

Previously, we identified specific host modulations in response to FMT and in the absence of FMT as well as microbial detection changes by treatment group. Identifying these host-microbe interactions can provide insight into pathways either on the host side or on the microbial side that are playing a role in inflammation outcomes. To determine likely interactions between host and microbe modulations, we employed a discriminant analysis using the mixOmics package in

R. Correlation cutoffs were set to 0.85 revealing strong linkages between a subset of host genes and MAGs (Figure 6). Two host genes displayed both positive and negative correlations between a set of MAGs: *CSAD* and *TRAFD1*. *CSAD*, as shown above to be upregulated in dysbiotic mice, was positively correlated to two MAGs: Enterobacteriaceae MAGs 167 and 169. This gene was also negatively correlated with several MAGs closely matching the families Lachnospiraceae and Oscillospiraceae. The *TRAFD1* gene, responsible for controlling immune response to pathogens, was positively correlated with Enterobacteriaceae MAG 169, and negatively correlated with multiple MAGs from the Lachnospiraceae and Oscillospiraceae families. One MAG of the *Dorea* genus displayed several positive correlations to the *Fut10*, *Wrap53*, *Primpol*, *Chd5*, and *Gnl1* genes. The trends overall indicate that Enterobacteriaceae MAGs are positively correlated with inflammatory and host amino acid metabolic response genes, while the Lachnospiraceae and Oscillospiraceae families are negatively correlated with inflammatory genes. Here, we show that bacteria from the Enterobacteriaceae family may drive host response in a dysbiotic gut through crosstalk with the host immune system and uptake of essential amino acids.

Metagenome-assembled genomes reveal potential microbial members impacting host IBD outcomes through sequestration of amino acids and nitrate

We used Pathosystems Resource Integration Center (PATRIC) web portal (Ondov et al. 2016; Davis et al. 2020) and constructed phylogenetic trees to further verify the identity of the MAGs. We verified that MAG 001 annotated to *Akkermansia muciniphila*, and MAG 002 was closely related to the *Barnesiella* genus in the Bacteriodota phylum. On the other hand, the other 5 MAGs were from two different taxonomic groups - *Enterococcus* (MAG 165 and 168) with the closest relationship to *Enterococcus faecium* and *Enterococcus faecalis* respectively, and Enterobacteriacaea (MAG 166, 167, and 169) had the closest phylogenetic relationship with *Enterobacter*, *Kosakonia*, and *Proteus*, respectively (Figure 7A). Out of the three groups of MAGs of interest, MAGs-*Akkermansia* was highly detected in all of the samples (control/no-CPZ: 0.894 ± 0.0496; CPZ/no-gavage: 0.997 ± 0.0005; CPZ/gavage-with-PBS: 0.924 ± 0.0505; CPZ/FMT: 0.998 ± 0.0005), regardless of whether the mice were dysbiotic or not. There was no statistical significance between the 4 treatment groups (t-test value=XXX, p=XXXX, Figure 7A). MAGs-*Enterococcus* and MAGs-Enterobacteriaceae had a very different detection pattern as that of MAGs-*Akkermansia* (Figure 7A). Both MAGs-*Enterococcus* and MAGs-Enterobacteriaceae displayed low detection in the control/no-CPZ (MAGs-*Enterococcus:* 0.005 ± 0.0015, MAGs-Enterobacteriaceae: 0.005 ± 0.0035) and CPZ/FMT (MAGs-*Enterococcus:*

0.002 ± 0.0005, MAGs-Enterobacteriaceae: 0.003 ± 0.0006) groups, however, were highly detected in the CPZ/no-gavage (MAGs-*Enterococcus:* 0.087 ± 0.0501, MAGs-Enterobacteriaceae: 0.057 ± 0.0226) and CPZ/gavage-with-PBS (MAGs-*Enterococcus:* 0.343 ± 0.0776, MAGs-Enterobacteriaceae: 0.303 ± 0.0583) group. We also observed a high statistical significant difference in the detection of MAGs-*Enterococcus* and MAGs-Enterobacteriaceae between mice that were treated with FMT as compared to mice received PBS or were not given any gavages the treatment groups (Figure 7A).

Several previous studies indicate the development of IBD could be associated with microbial factors including metabolic pathways driving host inflammation in addition to sustaining colonization, virulence factors such as secretion systems and effectors, and antibiotic resistance genes. We further assessed the potential gene functions in the 7 MAGs of interest (MAGs-*Akkermansia*, MAGs-*Enterococcus*, and MAGs-Enterobacteriaceae) to examine whether genome-level alterations related to those functions were observed in our resolved MAGs. We observed an absence of virulence genes that were detected in MAGs-*Akkermansia*; however, it included multiple antimicrobial resistance genes. On the other hand, MAGs-Enterobacteriaceae clustered on its own branch, with highly detected denitrifying gene clusters, T6SS, and multidrug efflux pumps. Some of the MAGs in both the groups MAGs-*Enterococcus* and MAGs-Enterobacteriaceae indicated sulfur metabolism functional potential whereas there were no MAGs in the group MAGs-*Akkermansia* displaying potential sulfur metabolism (Figure 7B).

Finally, potential metabolic pathways of MAGs-Enterobacteriaceae and MAGs-*Enterococcus* were analyzed to understand the metabolic activity in these MAGs that coincide and compete with host metabolism. KEGG pathway analysis revealed MAGs-Enterobacteriaceae contained sulfur gene pathways, involved in both biosynthesis and degradation of the amino acid cysteine (Figure 7C). By analyzing COGG potential genes, cysteine desulferases *SufS* was present in Enterobacteriaceae MAGs with *SufE* being present in Enterococcus MAGs, indicating the ability to utilize cysteine as a source of sulfur. The Enterobacteriaceae group also displayed the *TauD* gene for utilization of taurine as a source of sulfur as well. In addition to amino acid metabolism, the MAGs-Enterobacteriaceae group harbored a large presence of nitrate and nitrite reductases capable of denitrifying nitrate and nitrite to ammonia (Figure 7C). COGG genes from both MAGs-Enterobacteriaceae and MAGs-*Enterococcus* groups include the NarL and NarX gene families, a two-component system regulating various nitrate and nitrite reductases. Overall, this suggests that Enterobacteriaceae, and to an extent *Enterococcus*, compete with the host for

amino acids crucial to host homeostasis, and are able to utilize products like nitrate of the host immune response for growth and colonization of the dysbiotic gut environment.

Discussion

Early FMT leads to establishment of a persisting gut microbiota

In this study, we examine the efficacy and lasting impact of a series of FMT delivered via oral gavage at an early age, considering both host and microbial status. We showed that members of the donor microbiota had successfully established in the FMT-treatment mice and persisted until the end of the sampling period (Figure 2). We further showed that dysbiotic mice did not fully recover microbial diversity, and that mice receiving FMT were successful in recovering their microbial diversity and maintained throughout the 24-week study. Over 93% of the total non-redundant MAGs detected were Firmicutes, which are typically vulnerable to broad spectrum antibiotics like cefoperazone, thus the drastic depletion of detections would be expected in dysbiotic mice (27). Our data showed that indeed without exposure to a donor microbiota, numerous MAGs that were annotated to Firmicutes were missing in mice in the dysbiotic groups, receiving no gavage or saline gavage by the endpoint of the study (28). As compared, we observed that mice receiving FMT had a higher number of MAGs that were annotated to Firmicutes, which were missing in the other two dysbiotic groups, suggesting FMT during development had a lasting impact on microbial makeup until the endpoint of the study. Our analysis revealed differential detection of the resolved MAGs between the treatment groups, but are there any specific microbial populations that were associated with IBD pathogenesis, and what are the niche functional specificity for these differentially detected MAGs?

Specific microbes and their involvement in inflammation and IBD

We highlighted in our study that not only were there distinct differences in the microbial community among the treatments, but there were also specific microbial populations that were associated with the positive and negative outcomes of FMT (Figure 3). Microbial communities and diversity are important in maintaining gut homeostasis, however specific microbes can impact the outcome of IBD in both positive and negative ways. In our study, dysbiotic mice receiving the saline gavage or no gavage, showed higher detection averages of the MAGs-*Enterococcus* and MAGs-Enterobacteriaceae than control mice or mice receiving FMT (Figure

3). We surmise that these two classes of MAGs were colonizing the dysbiotic gut and are involved in the increased inflammation found in these mice compared to other treatment groups.

We found that our resolved MAGs-*Enterococcus* harbored several multidrug efflux gene functions, which are known aggravators of colitis and Crohn's disease (14, 29). Furthermore, the presence of Type VI Secretion Systems (T6SS) in MAGs-*Enterococcus* could worsen the onset of colitis (15, 30). Our results corroborated with other previous findings suggesting that strains of commensal *Enterococcus* can also impact other instigators of inflammation, such as *Escherichia coli*, and can activate defense systems like the Type III secretion system (15, 30). On the other hand, MAGs-*Enterococcus* might also have the capacity to function as a probiotic and possibly plays a role in lowering inflammation due to butyrate metabolism and antioxidant activity (31, 32).

In this study, our resolved MAGs-Enterobacteriaceae were highly detected in dysbiotic mice, suggesting that these MAGs are be implicated in worsening inflammation and overgrowing in the colon (12, 13, 33), exacerbating symptoms and development of IBD. Due to a wide range of bacterial strains residing in this family, our MAG-Enterobacteriaceae display a complex association with IBD pathogenesis. For example, *Escherichia coli* is a widely diverse species of commensals and pathogens (34, 35), and have been associated with increased inflammation and severity of IBD. We found denitrifying gene clusters in our resolved MAG-Enterobacteriaceae that have been implicated with the virulence factors (12, 34) and metabolic pathways (36, 37) of strains of *E. coli* that are associated with increased colon inflammation. In addition, Enterobacteriaceae MAGs indicate a presence of several cysteine and taurine dioxygenases and transporters which are responsible for taking in cysteine and taurine from the environment and breaking them down for a source of sulfur. This combined with the host gene CSAD being significantly upregulated in dysbiotic mice, allow us to suspect that these Enterobacteriaceae members are competing with the host for sulfur rich amino acids, exacerbating host inflammation and IBD symptoms (21).

Here, we showed that MAGs-*Enterococcus* and MAGs-Enterobacteriaceae might play interesting and oftentimes contradictory roles in the development or prevention of IBD, providing evidence that even microbes of the same species have complex and multifaceted relationships with the human gut. However, we also showed that MAGs-*Enterococcus* and MAGs-

Enterobacteriaceae, and their potential virulence functions could be a crucial indicator in identifying colon inflammatory conditions (38).

Our resolved MAGs-*Akkermansia* group consisted of a MAG (MAG 001) that was identified as being related to *Akkermansia muciniphila* and another MAG (MAG 002) that was closely related to *Barnesiella.* We observed several antibiotic resistance associated genes in these two MAGs which explained the high detection of the MAGs-*Akkermansia* in all mice, regardless of antibiotic status or treatment. MAG 001 had gene functions that were associated with mucin degradation which had been suggested to be reasons in maintaining gut epithelial homeostasis and integrity (17, 39, 40), and thus been proposed as a widely accepted probiotic species. We postulate the mucin degradation and probiotic characteristics of MAG 001 could be one of the factors why there were few incidences of extreme colitis among this cohort of mice in this study, although it did not prevent colon inflammation in dysbiotic mice entirely. The role of MAG 002 in reducing inflammation in colitis (41) was a bit more complicated, with gene functions that were associated with protection against potential infections in the intestines (42), regulating intestinal homeostasis and interacting with the immune system (41, 43)). In summary MAGs-*Akkermansia* were likely able to alleviate symptoms of colitis while maintaining normal gut conditions, even though they were not able to completely alleviate inflammation in this study.

In this study, we demonstrated the importance of re-introducing a healthy microbial community via FMT early in development to prevent the onset of IBD later in life. Mice receiving FMT displayed lower inflammation in the colon, higher detections of more MAGs at the endpoint of the study, and gene regulation patterns similar to control mice. The early colonization of the microbial community had long-term impacts on the microbiota status in these mice, suggesting that development is a crucial time point for establishment of a diverse microbiota to prevent chronic inflammatory diseases such as IBD. Establishing that the early interactions between host and microbes in the intestines are important for host homeostasis, immune development, and prevention of inflammatory diseases. This research has identified groups of microbial members that potentially influence the outcomes of disease positively and negatively. In addition, this work has identified several metabolic pathways potential pathogenic microbes use to steal essential nutrients from the host while fueling their growth and over colonization. Further research into the mechanism of these interactions between microbes and the host are needed in developing therapeutics for combating the overgrowth of Enterobacteriaceae and *Enterococcus* leading to inflammatory disorders like IBD. Studying individual bacterial members

in the *Enterococcus* or Enterobacteriaceae groups detected this study could shed light on how typically commensal microbes adapt to over colonize and spark inflammatory responses, likely triggering IBD or related conditions. Understanding pathways of pathogenesis for complex diseases such as IBD can provide a foundation in understanding the microbial functions and environmental alterations needed for potential treatments and therapeutics.

Materials and Methods

Sample collection and processing

IL-10 KO Mice. All mice in this study were IL10 KO Jax stock #002251/ C57BL/6J genetic background. Mice were bred in the Kansas State University Johnson Cancer Research Center mouse facility under *Helicobacter hepaticus*-free conditions. Once dams were in the third week of gestation, autoclaved drinking water was supplemented with (0.5 mg/mL) of cefoperazone sodium salt (CPZ), a broad-spectrum antibiotic. The duration of CPZ exposure lasted until pups were weaned at 21 days of age. Fecal samples were collected from control mice and were frozen to -80□. To prepare the slurry for gavaging, control fecal samples were thawed, pooled, and gently vortexed in sterile PBS, then immediately frozen to -80□ until use. By week 23, remaining mice received 2% DSS for 5 days in autoclaved drinking water and was changed as needed. Mice were monitored daily during the time of DSS treatment and after treatment, until the end of the study. After a 16-day recovery period after DSS treatment, all mice were sacrificed, and tissues were collected.

Ethics Statement. All mouse experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Kansas State University (APN #4391).

Serum. Blood was collected via heart puncture and centrifuged to collect serum. Serum was stored in -80□ until further analysis. Mouse Cytokine 31-Plex assay (Eve Technologies, Canada, catalog: #33836) measured Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, and VEGF-A (not validated).

Histopathology. A small portion of the lower large intestine from each mouse were fixed in 10% neutral-buffered formalin. Paraffin embedded cross-sections were stained with hematoxylin and eosin and blindly scored by an anatomic pathologist (Dr. Brandon Plattner, College of Veterinary Medicine, Kansas State University, Manhattan, KS) for characteristics of inflammation epithelial damage, infiltrating cells, and crypt damage indicating IBD status. Grading was broken into four categories, inflammation, surface damage, crypt damage, and crypt hyperplasia, each being on a scale from 0-3 by severity. Summative scores for each mouse and means for each treatment group were calculated.

Shotgun Metagenomics. Pups were tracked for 23 weeks. Fecal samples were collected at week 24 and immediately frozen to -80□ until extraction was performed. gDNA was extracted using the E.Z.N.A Stool DNA Kit using the manufacturer protocol for Pathogen detection supplemented with 10 minutes of bead beating. Extracted DNA was kept at -20□ until shotgun metagenomic sequencing. Sample DNA concentration and quality were determined by Qubit and Nanodrop. DNA libraries were constructed using Illumina's Nextera XT Index Kit v2 kit. The products were then visualized on an Agilent Tapestation 4200 and size-selected using the BluePippin. The final library pool of XX samples was quantified on the Kapa Biosystems qPCR protocol and sequenced on the Illumina NovaSeq S1 chip in a 2 x 150bp paired-end sequencing run.

Colon RNA Sequencing. A small portion of the lower large intestine were stored in TRIzol reagent and stored at -80□ until sequencing. Samples were sent to CD Genomics, where they were extracted, and QC analysis performed by manufacturer protocols. Next, cDNA library preparation (poly-A selection) was completed for the 12 samples and were then sequenced via NovaSeq PE150 at around 20M.

Bioinformatic workflow

Metagenomics. We automated our metagenomics bioinformatics workflows using the program ‘anvi-run-workflow’ in anvi’o. The workflows use Snakemake and implement numerous tasks including short-read quality filtering, assembly, gene calling, functional annotation, hidden Markov model search, metagenomic read-recruitment and binning.

We used the program ‘iu-filer-quality-minoche’ to process the short metagenomic reads, and remove low-quality reads according to the criteria outlined by Minoche et al. We used MEGAHIT v1.2.9 to assemble quality-filtered short reads into longer contiguous sequences (contigs). We then used the following strategies to process the assemblies: we used (1) ‘anvi-gen-contigs-database’ on contigs to compute k-mer frequencies and identify open reading frames (ORFs) using Prodigal v2.6.3; (2) ‘anvi-run-hmms’ to identify sets of bacterial and archaeal single-copy core genes using HMMER v.3.2.1; (3) ‘anvi-run-ncbi-cogs’ to annotate ORFs from NCBI’s Clusters of Orthologous Groups (COGs); and (4) ‘anvi-run-kegg-kofams’ to annotate ORFs from KOfam HMM databases of KEGG orthologs.

We mapped metagenomic short reads to contigs using Bowtie2 v2.3.5 and converted to BAM files using samtools v1.9. We profiled the BAM files using ‘anvi-profile’ with minimum contig length of 1000bp. We used ‘anvi-merge’ to combine all profiles into an anvi’o merged profile for all downstream analyses. We then used ‘anvi-cluster-contigs’ to group contigs into initial bins using CONCOCT v1.1.0 and used ‘anvi-refine’ to manually curate the bins based on tetranucleotide frequency and different coverage across the samples. We marked bins that were more than 70% complete and less than 10% redundant as metagenome-assembled genomes (MAGs). Finally, we used ‘anvi-compute-genome-similarity’ to calculate average nucleotide identity (ANI) of our genomes using PyANI v0.2.9 and identified non-redundant MAGs.

We used the “detection” metric to assess the occurrence of MAGs in a given sample. Detection of the MAGs is the proportion of the nucleotides that were covered by at least one short read. We considered a MAG was detected in a metagenome if the detection value was > 0.25, which is an appropriate cutoff to eliminate false-positive signals in read recruitment results, for its genome. We used ‘anvi-gen-variability-profile’ with ‘--quince-mode’ to export single-nucleotide variant (SNV) information on all MAGs after read recruitment, to identify subpopulations of the MAGs in the metagenomes. We used DESMAN v2.1.1 to analyze the SNVs and determine the number and distribution of subpopulations in the MAGs. We accounted for non-specific mapping by removing any subpopulations that made up less than 1% of the entire population that were explained by a single MAG.

PATRIC analyses. Non-redundant genomes were uploaded and annotated in Pathosystems Resource Integration Center (PATRIC). For the 7 MAGs of interest, similar genomes were identified, and phylogenetic trees were built using 50 similar strains to identify close

relationships and approximate identity. Next, for each MAG Subsystem genes were utilized for comparison of potential functions and virulence factors between the 7 MAGs. Counts of genes by each class of interest were used to build a heatmap and pathway constructs for each MAG of interest by using the Pathway function in PATRIC linked to KEGG.

RNASeq analysis. The quality of raw reads were checked using FastQC (44) and multiQC (45), and subjected to quality control with Trimmomatic (46). Trinity (47) was used for de novo assembly. RSEM software (48) and DESeq2 (49) were used to estimate the expression levels and differential expression analysis, respectively. Annotation was carried out using blast+ 2.11 and R version 4.0.0. The transcripts compared in DESeq2 were first blasted against mouse transcript with C57BL/6NJ as a reference ensemble ID (http://ftp.ensembl.org/pub/release-107/fasta/mus_musculus_c57bl6nj/cdna/), then the top hit (lowest e-value and largest length with minimum of 150bp) were kept. GO_MWU analysis (50) were performed on the annotated transcripts.

Statistical analyses. Differences between groups were analyzed by unpaired Student's t-test, unless stated otherwise. P-values of less than 0.05 were considered statistically significant. Statistical analyses were performed in GraphPad Prism 9 or RStudio.

Data availability

Accession Number. Raw sequencing data for the mice metagenomes were uploaded to SRA under NCBI BioProject PRJNA843079.

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Conflict of Interest

The authors disclose no conflicts of interest.

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