Enhancing Recovery from Gut Microbiome Dysbiosis and Alleviating DSS-Induced Colitis in Mice with a Consortium of Rare Short-Chain Fatty Acid-Producing Bacteria

Achuthan Ambat[1, 2†], Linto Antony[1†], Abhijit Maji[1], Sudeep Ghimire[1], Samara Mattiello[1], Purna C. Kashyap[3], Vanessa Sebastian[4], Joy Scaria[1, 2*]

[1]Department of Veterinary and Biomedical Sciences, South Dakota State University, Brookings, SD USA

[2]Department of Veterinary Pathobiology, Oklahoma State University, Stillwater, OK USA

[3]Enteric Neuroscience Program, Department of Medicine and Physiology, Mayo Clinic, Rochester, Minnesota, USA

[4]Department of Pathology, Jubilee Mission Medical College and Research Institute, Kerala, India

[†]Equally contributing authors

*** Correspondence:**
Corresponding Author
joy.scaria@okstate.edu

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Abstract

The human gut microbiota is a complex community comprised of hundreds of species, with a few present in high abundance and the vast majority in low abundance. The biological functions and effects of these low-abundant species on the host are not yet fully understood. In this study, we assembled a bacterial consortium (SC-4) consisting of *B. paravirosa*, *C. comes*, *M. indica*, and *A. butyriciproducens*, which are low-abundant, short-chain fatty acid (SCFA) producing bacteria isolated from healthy human gut and tested its effect on host health using germfree and human microbiota associated mice models of colitis. Our findings demonstrate that SC-4 can colonize in Germ-free (GF) mice, increasing mucin thickness by activating the MUC-1 and MUC-2 genes, thereby protecting GF mice from Dextran Sodium Sulfate (DSS)-induced colitis. Moreover, SC-4 aided in the recovery of human microbiota-associated mice from DSS-induced colitis, and intriguingly, its administration enhanced the alpha diversity of the gut microbiome, shifting the community composition closer to control levels. We also show a functional redundancy existing in the gut microbiome, resulting in the low abundant SCFA producers acting as a form of insurance, which in turn accelerates recovery from dysbiotic state upon the administration of SC-4. SC-4 colonization also upregulated iNOS gene expression, further supporting its ability to enhance mucin thickness upon colonization. A metagenomic analysis of Inflammatory Bowel Disease (IBD) patient samples revealed a decrease in the abundance of SC-4 bacteria in both Ulcerative Colitis (UC) and Crohn's Disease (CD), highlighting the potential importance of these species in the human gut. Collectively, our results provide evidence that low-abundant SCFA-producing species in the gut may offer a novel therapeutic approach for IBD.

Keywords: Microbiome, Colitis, Short chain fatty acid, Butyrate, Low abundant species, Defined bacterial therapy, Insurance species

Introduction

In biological ecosystems, species composition typically follows a skewed pattern, wherein a small subset of species are highly abundant while the majority are low in abundance or relatively rare. This phenomenon, first observed by Darwin in his groundbreaking work[1], is a shared characteristic across diverse biological classes and geographies. The same skewed distribution was later recognized by Preston in bird and moth populations during the 20th century[2].This principle of skewed biodiversity distribution is also reflected in the composition of the human gut microbiota. An analysis of gut microbiome composition from individuals across various continents revealed a bimodal species distribution[3]. In this distribution, a handful of highly abundant species exist on the left of the log-normal distribution, while a large number of rare species congregate on the right. Upon further exploration of species distribution across thousands of individuals, Lawley and colleagues discovered that the human gut microbiota is primarily dominated by about 20 species, with the remaining species present in lower abundance[4].

As a result, the focus of human gut microbiome research has been primarily directed towards understanding the role and implications of these dominant species. Numerous studies have found that these highly abundant species often play vital roles in gut functionality. One such species is

Bacteroides thetaiotaomicron, a common inhabitant of the human gut. Extensive research on this organism has provided valuable insights into how dominant members of the microbiome can contribute to maintaining health and wellbeing. *B. thetaiotaomicron* plays a key role in nutrient utilization in the gut where it helps to metabolize polysaccharides otherwise inaccessible to the host[5]. Another well understood example of high abundant gut species is *Faecalibacterium prausnitzii* which has been recognized as one of the main butyrate producers found in the intestine[6]. High abundant species also could be problematic. A well known example for that is *Escherichia coli* which depending on the strain type could proliferate in the dysbiotic gut can cause mild to fulminant diarrhea[7].

Metagenomic investigations of the gut microbiome have unveiled several thousand species spanning diverse populations, with the majority identified as low-abundance species. Intriguingly, most of these rare species have not been cultured, and even among those that have been, comprehensive functional and mechanistic studies are sparse. Despite possessing a reasonable understanding of certain dominant species residing in the gut, our knowledge regarding the role of these rare species remains largely under-explored. Emerging research, however, indicates that these low-abundance species may profoundly influence host health, serving as potent activators of gut immunity, among other potential effects. Eventually these species contribute to the total richness and diversity of the system. Contrary the loss of diversity have shown association with various enteric and systemic diseases [8], further suggesting to the fact that these species might have significant role in protection against these diseases.

In non-microbial communities, higher rates of extinctions have motivated interest in diversity-stability relationships [9-12]. Insurance hypothesis states that high species richness reduces temporal variability of a given community properties by insuring the community against various perturbations [13]. The same concept has seen to be existing in microbial communities also. For example Lieven, et al has shown in experimental microcosms that the degree of richness and evenness in a community prior to perturbance affects the subsequent response to the perturbation [14]. Later, the same was explained in the case of conditionally rare taxa (CRT) in gut microbiome [15].

The objective of our study was to delve deeper into the functional roles of rare species in the gut, particularly those capable of producing short chain fatty acids (SCFAs). SCFAs were selected as the focal point due to their significant contribution to immune cell proliferation[16], differentiation[17], apoptosis[18], gut barrier integrity[19], gut motility[20], and host metabolism [21].Among the characterized SCFAs, butyrate has demonstrated protective effects against immune disorders, such as Intestinal Bowel Disorders (IBD) [22]. Although direct administration of butyrate compounds as a therapeutic intervention against IBD has encountered obstacles related to delivery, exposure duration, and patient compliance, there is a continued interest in the beneficial properties of butyrate[23, 24]. The success of using prebiotics as an IBD treatment hinges on the presence of butyrate-producing bacteria[25]. For instance, treatment with butyrate-producing *F. praunitzi* was found to rebalance the aberrant microbiota community in an IBD mouse model, while *Butyricicoccus pullicaecorum* led to the attenuation of colitis in rats[26]. Given the scarcity of functional studies on rare gut species, we employed germfree (GF) and humanized mouse models to explore the interspecies interactions of a manageable consortium of four SCFA-producing rare species in the human gut. We monitored changes in the gut microbiome and host responses. Our findings indicate that the rare species mix we utilized colonized GF mice and alleviated DSS-induced colitis. In humanized mice, the same mix expedited the recovery of microbiome beta diversity and enhanced host health following DSS perturbation. In essence, our study illuminates the significant functional roles that rare species can play in the gut, suggesting potential avenues for improving gut health using these rare species.

Results

Selection of strains for Short Chain fatty acid producing consortium (SC-4)

In our previous work, we developed a microbiota culture collection from healthy human donors that accounted for over 70% of the functional capacity of the healthy gut microbiome[27]. This collection included both high-abundance and rare species. To curate an assembly of short-chain fatty acid (SCFA) producing strains from this collection, we focused on the ability to produce butyrate, an SCFA with substantial beneficial impacts on the host. We relied on both experimental inference and extant literature for this purpose. Given that several genes responsible for butyrate production in bacteria are well-documented, we utilized the presence of these genes as the primary criterion for species selection in this study.

Additionally, we determined the percentage abundance of butyrate-producing bacteria in the donor fecal samples used for developing our culture collection[27] **(Fig. 1A)**, as well as in a publicly available healthy human shotgun metagenomics dataset[28] **(Fig. 1B)** [27, 29-32]. Aligning with prior reports, our analysis revealed that the well-known butyrate-producing species *F. praunitzi* was highly abundant in the donor metagenomes. Nevertheless, the mean abundance of most putative butyrate-producing species was less than 0.1%. To constitute a manageable mix of strains for further study, we randomly selected four species with an abundance below this threshold **(Supplementary fig 1A)**. The abundance of these selected strains measured from the metagenome sequence data of six donor fecal samples showed that all four bacteria exhibited a mean abundance of less than 0.1% **(Fig. 1A & B, Supplementary Fig 1A & B)**.

The relative abundance of the presumptive butyrate-producing species in our dataset aligned with the relative distribution across analyzed public datasets **(Fig. 1B, Fig. 5F-I)**. The genotypic and phenotypic potentials of the selected strains to produce butyrate served as criteria for their selection. To confirm their genotypic potential, their genome sequences[33] were scrutinized using BLAST to identify markers of the butyrate synthesis pathway, specifically butyryl-CoA: acetate CoA transferase (encoded by *but*) or butyrate kinase (encoded by *buk*). These two represent the terminal genes in the most prevalent pathway of butyrate production in the gut microbiota[34]. *Butyricimonas paravirosa* and *Coprococcus comes* exhibited the presence of *but*, while *Megasphaera indica* and *Agathobaculum butyriciproducens* demonstrated the presence of *buk* in their genome **(Supplementary Fig. 1C)**. Their ability to produce butyrate and other SCFAs was confirmed by *in vitro* culturing of the strains in three different media, followed by gas chromatography estimation of SCFAs in the culture supernatant **(Supplementary. 1D)**.

Estimation of intra-consortium interaction of SC-4 *in vivo*

One hypothesis for a species being rare within a community is its competition with other species. To assess whether the chosen rare species could stably colonize the host gut and to investigate the interactions between these low-abundance species, we employed a germ-free mouse model to poly-associate the SC-4 consortium. After administering equal proportions of the SC-4 mix orally via gavage, we collected fecal samples at different time points to assess the bacterial load. An average bacterial load of $>10^7$ cfu/g feces was detected from the 4th day post-inoculation (DPI), which continued to increase until it reached $>10^8$ cfu/g feces **(Supplementary Fig. 2A)**. During these time points, we didn't observe any significant drop in bacterial load, suggesting successful and stable colonization of the SC-4 consortium in the mouse gut. An average bacterial count of nearly 10^9 cfu/g

in the cecal content **(Supplementary Fig. 2B)** further verified their successful colonization in this allochthonous host species.

Community profiling via 16s rRNA gene amplicon sequencing revealed the presence of all four bacteria, although their abundance varied and remained consistent throughout the experiment **(Fig. 2A, Supplementary 2C-E)**. We found a similar community structure in the cecum and colon, but we did not obtain any amplification for 16s PCR from small intestine contents **(Fig. 2A, Supplementary 2C)**. This demonstrates the consortium's ability to colonize the distal gut of germ-free mice. *C. comes* had a higher mean abundance in all the mice, while *A. butyriciproducens*, *B. paravirosa*, and *M. indica* displayed relatively lower abundance in colonized mice. Next, we examined whether the colonized consortium could produce butyrate and other short-chain fatty acids (SCFAs). As anticipated, the colonized consortium was capable of producing butyrate and other SCFAs. Analysis of cecal content using gas chromatography (GC) detected significantly higher levels of all key SCFAs, except acetate, in the SC-4 colonized mice compared to germ-free mice **(Fig. 2B)**.

Colonization of SC-4 modulates the host immune system

To explore how the consortium interacts with the host, we analyzed the expression profile of a panel of 91 genes in the colon using quantitative Real-Time PCR (qRT-PCR). When compared to germ-free (GF) mice, nine genes showed more than two-fold change differences in their expression in the SC-4 colonized group **(Fig. 2C)**. Colonizing GF mice with a potential pathogen usually increases the expression of various pro-inflammatory and anti-inflammatory cytokines, as well as Toll-Like Receptors (TLRs), in the intestine [35] The absence of significant changes in the expression levels of these genes and intestinal barrier proteins in the colon suggests that the SC-4 species do not negatively impact the host, even when colonized at high levels. Notably, changes in expression were found primarily in genes involved in the host's initial immune defenses, including mucins (MUC-1 and MUC-2) and antimicrobial peptides (Reg3b, Reg3g, retnlb, and Defb37). Interestingly, SC-4 colonization also increased the expression of Fatty Acid Binding Protein 2 (FABP2/I-FABP) and one of the G-protein coupled receptors (GPRs) - FFAR2 or GPR43 **(Fig. 2C)**. FABP2 is a cytosolic protein that binds Free Fatty Acids (FFA) and is thought to be involved in the uptake and intracellular trafficking of lipids in the intestine. Conversely, FFAR2 is a receptor for short-chain fatty acids [36]. We believe that since the colonized members of the SC-4 were capable of producing butyrate in the mouse gut, and butyrate is an energy source for the colonocytes, the expression of insulin-like peptide 5 (Insl5) showed more than two-fold downregulation.

To better understand the adaptive immune changes elicited by the colonization of SC-4, we profiled total lymphocytes (CD45+) collected from both secondary lymphoid organs (spleen) and peripheral blood. Flow cytometry analysis of major adaptive immune cell populations, such as B-cells (CD19+), γδT cells, and subsets of ∞βT cells - T-helper (CD4+), T-cytotoxic (CD8+), and Th17 (RORγ+) showed noticeable differences in the adaptive immune phenotypes between GF and SC-4 colonized mice. Interestingly, the percentage of B cells and ∞βT cells in the total lymphocytes were significantly lower in the spleen of SC-4 colonized mice **(Fig. 2D-F)**. Within ∞βT cells, there was a significant decrease in the CD8+ T cells and an increase in CD4+ T cells **(Fig. 2G,H)**. However, the percentage of Th17 cells within CD4+ T cells was significantly reduced in GF mice **(Fig. 2I)**.

In peripheral blood, ∞βT cells showed a similar trend as in the spleen, but there were no significant differences in the proportions of CD4+, CD8+, and Th17 cells compared to GF mice. However, Th17 cells showed a decrease in proportion within CD4+ T cells, even though this was not statistically significant **(Fig. 2n)**. Contrary to the spleen, B cells and γδT cells were significantly increased in BP4

colonized mice **(Fig. 2d-o)**. Histopathological analysis of colon tissue after H&E staining showed normal appearance in both GF and BPC colonized gnotobiotic mice without any disruption in mucosal architecture **(Fig. 2p)**. However, some morphological changes were observed between the two groups. Both villus length and crypt depth were increased in gnotobiotic mice compared to GF controls. The submucosa and muscularis propria were thicker in gnotobiotic mice than GF mice, but without any pathological signs such as submucosal edema. We believe this could be connected with the increase in expression of MUC-1 and MUC-2 genes when the GF mice are colonized with the SC-4 consortium.

SC-4 colonization protects GF mice from DSS-induced colitis

Since the colonization of SC-4 induced positive immune changes in the GF mice, we further examined whether this modulation could provide protection against disorders such as IBD. To determine this, SC-4 pre-colonized mice were treated with DSS to induce colitis and monitored throughout the experiment **(Supplementary. 3A)**. Mice were sacrificed on day 21 and the community formed in the colon and cecum was assessed using 16s rRNA sequencing. The community showed the same trend as what we have previously observed with SC-4 colonization **(Fig. 3A)**. SC-4 colonization also protected mice against colitis induced death (Sup B). *C. comes* relative abundance was slightly decreased with an increase in abundance for the rest of the three bacteria (Fig. 3A). This suggests that *C. comes* may have an antagonist effect against the other three bacteria. This trend remained true for both the colon and cecum **(Fig. 3A, Supplementary. 3C)**. Further, quantification of short-chain fatty acids showed no difference when treated with DSS **(Fig. 3B)**. There was a slight increase in the production of butyrate. This implies that the induction of a disease state did not reduce SC-4 ability to produce SCFAs.

Colitis in mice is often characterized by noticeable changes such as shortening of the colon. Interestingly, our results showed that SC-4 pre-colonized mice exhibited less colon shrinkage compared to the control group **(Fig. 3C)**, suggesting that SC-4 pre-colonization may offer systemic protection against DSS-induced colitis. Further we examined differential gene expression upon disease induction. However, compared to control, SC-4 mice treated with DSS did not show any biologically relevant changes **(Fig. 3D, E)**. We also checked for the systemic Immune response; apart from ∞βT cells, there were no significant changes in the cell profile in DSS-treated animals **(Fig. 4A-I)**. There was a substantial reduction in the ∞βT cell population in both PBMC and spleen for SC-4 pre-colonized mice **(Fig. 4B, H)**. This result is comparable to the result previously observed when GF mice are colonized with SC-4 **(Fig. 3E, K)**. However, no significant TH17 cells were increased in the spleen nor PBMC of GF mice with colitis. Our assumption for the change could be the previous recognition of molecular patterns of the SC-4 consortium. Finally, Histopathology analysis of GF mice induced with colitis showed moderate inflammation, loss of goblet cells, vascular proliferation as well as wall thickening, **(Fig. 4M)** but when pretreated with SC-4 **(Fig. 4N)** showed no to mild inflammation in most mice. All of them showed a normal or slight increase in mucosa and architecture. Since we did not identify substantial differences in systemic immune response, we hypothesize that the protective effect conferred by SC-4 might be attributed to improvement of gut barrier function primarily through an increase in expression of the MUC-1 and MUC-2 genes. These genes, which contribute to the thickness of the submucosa and muscularis propria, may enhance barrier function.

SC-4 members show a negative correlation with colitis in Humanized mice

Next, we examined whether SC-4 consortium could colonize mice, establish higher abundance and modulate microbiome composition and as well as improve host health in the presence of complex human microbiota. To this end, we gavaged SC-4 consortium in human fecal microbiota colonized mice. The composition of microbiome following SC-4 gavage was analyzed by 16s rRNA sequencing of the fecal pellets. While *Butyricimonas paravirosa* (Odoribacteraceae), *Coprococcus comes* (Lachnospiraceae) and *Agathobaculum butyriciproducens* (Ruminococcaceae) were detected in the community **(Fig. 5A, 5G)**, *Megasphaera indica* (Veillonellaceae) was not detected in any of the mice. This indicates that these three bacteria can colonize the mouse gut even when introduced as a part of a complex community. Further, we examined whether the abundance of these three species changed during colitis . To this end, we induced colitis in SC-4 colonized humanized mice. The abundance of all three species was significantly reduced on the 6th day post-gavage **(Fig. 5A)**. However, the reduction in abundance reverted significantly on the 28th day post-gavage when the mice started to recover from colitis **(Fig. 5A)**. This indicates a negative correlation between these three species and colitis in mice. Since the abundance of these bacteria increased with time post-gavage, we hypothesized that the introduction of these species back into the system could help accelerate the recovery against DSS-induced colitis **(Supplementary. 4A)**.

Since fermentation of dietary fiber has been shown to promote the abundance of SCFA producing species, we examined whether addition of dietary fiber could increase the abundance of SC-4 and provide better protection against colitis. To this end, above set of experiments were repeated with and without inulin, a commonly used dietary fiber.

We examined the microbiome composition following these experiments and we found that the introduction of SC-4 increased the alpha diversity of the gut community in DSS-treated mice, whereas it was less in the control mice **(Fig. 5B, Supplementary. 5A-D)**. When the beta diversity between different groups were examined, we observed that SC-4 administration after colitis induction shifted the diversity to resemble to that of healthy humanized mice **(Fig. 5B, Supplementary. 5E-H).** This suggests that introducing SC-4 can increase the diversity and shift the microbiome to a control state. Besides the fact that SC-4 increases diversity, it also showed an increase in colon length, suggesting a higher rate of recovery for DSS-induced colitis **(Fig. 5F, Supplementary 5I)**. Gut bacteria utilize dietary fibers to produce SCFAs. We hypothesized that supplementing inulin to the diet with SC-4 can alleviate the response. As expected, the addition of Inulin increased the alpha diversity at a much higher rate **(Fig. 5C)**. For beta diversity analysis, as seen previously with SC-4, colonization of SC-4 with Inulin also clustered more towards control mice **(Fig 5E)**. But inulin and inulin with SC-4 administration in control mice had a different clustering which is expected because of the effect of inulin on the microbiome **(Fig. 5C)**. This also proves that Inulin alone is less efficient in increasing the diversity compared to SC-4 supplementation. Further addition of inulin with SC-4 showed an increase in colon length compared to treatment with Inulin **(Fig 5F)**. Since we know that a stable microbial community is functionally redundant, we want to check whether DSS treatment showed this redundancy in the humanized mice . To this end, we created a custom database of full length 16s rDNA for the known SCFA producing bacterial species as in **(Fig 1A-B)** and checked their abundance in the various treatments. This analysis showed a decrease in most of the high abundant SCFA producing bacteria upon DSS administration while low abundant bacterial species such as *Clostridium innocuum, Anaerostipes hadrus, Clostridium tyrobutyricum, and Hungetella hathewayi* increased in abudance **(Fig 5G)**. Further in the case of SC-4 as well as SC-4 along with Inulin treatment, the supplementation appears to increase in the rest of the SCFA producers in the community **(Fig 5G)**. Interestingly in both treatments we also found an increase in abundance of *Akkermansia mucicniphila* a know butyrate producing species with known gut health improving function. This suggest that SC-4 administration

might help in regaining the SCFA production through interspecies interactions that boost other beneficial species.

We checked the gene expression changes in mice colon upon colonization of humanized mice with SC-4 consortium. As noticed in the case of GF mice, we didn't see any significant changes in the inflammatory-related gene expression, further proving the non-pathogenic nature of the SC-4 consortium **(Supplementary. 5J-l)**. iNOS-dependent increase in colonic mucus has been demonstrated previously to exist in colitis-induced rats. We saw a significant increase in the expression of iNOS when colitis-induced mice were treated with SC-4 or SC-4 supplemented with Inulin **(Fig. 6A-C)**. We think SC-4 might be helping the mice to protect against more damage from colitis by increasing mucus production by the increase of iNOS. There was also a reduction in the expression of IL-6 compared to colitis recovered naturally. Myeloperoxidase (MPO) produces hypohalous acid to carry out the antimicrobial activity and was seen to be upregulated in colitis recovered naturally **(Fig. 6A)**. This might be because of the perturbation and community shift due to DSS-induced colitis, which increased the abundance of some pathogenic bacterial classes and hence the response. The same was downregulated when treated with SC-4 or SC-4 supplemented with Inulin. This indirectly shows the ability of the consortium to change the community into a healthy state. Histopathology of the colon section showed several differences between BP-treated mice and no treatment. Colitis-induced humanized mice **(Fig. 6D)** showed a similar phenotype as GF mice with colitis whereas when treated with SC-4 **(Supplementary. 6B)** or SC-4 supplemented with Inulin **(Fig. 6E)** showed a similar phenotype as control mice **(Supplementary. 6A)**. No to mild inflammation was observed. Normal to a slight increase in mucosal architecture, goblet cells, and crypts were seen. The muscular wall was also thin. These results suggest the ability of SC-4 to treat DSS-induced colitis and help to recover the abrupted microbiome.

SC-4 showed an inverse association with disease conditions.

Depletion or enrichment of a bacterial species in a disease condition might indicate the role of that species of bacteria in the prevention or exacerbation of the disease respectively. Nonetheless, this association doesn't prove their causal relationship to the disease. Thus, to know the association of selected species in this study to IBD, a disease prevalent in both developed and developing countries [37], the abundance of those bacteria was assessed in fecal metagenomes collected from both IBD and non-IBD individuals as a part of a longitudinal cohort study conducted by Lloyd-Price et al.[28].We checked the abundance of all four species in 598 metagenomes from 50 CD subjects and 375 metagenomes from 30 UC subjects. We compared it against the abundance in 365 metagenomes from 27 non-IBD subjects. Interestingly, the analysis showed the presence of all four bacterial families in both conditions (healthy and disease) with a mean abundance <0.1% **(Fig. 6F-I)**. Compared to non-IBD microbiota, all four bacteria showed a differential abundance in at least one of the IBD conditions. There were no statistically significant differences obsereved for any of the selected bacteria. But the median abundance of *Agathobaculum butyriciproducens, Megasphaera indica,* and *Butyricimonas paravirosa* were found to be decreased in both UC and CD patients **(Fig. 6G-I)**. We didn’t find an appealing difference in the median abundance for *Coprococcus comes* in both UC and CD patients. We think the lack of statistical confidence is because of the increase in sample size and interpersonal variation noticed among the individuals. These results confirmed that there is a reduction of the SC-4 bacteria in the gut of IBD Patients, indirectly pointing to the fact that SC-4 bacteria have a negative correlation with the IBD condition.

Discussion

In various biological systems, such as terrestrial, aquatic, and animal gut microbiomes, a skewed distribution of species abundance is often observed. Within the healthy human gut microbiome, this phenomenon is particularly pronounced: while hundreds of species exist, only a few are present in high abundance, and the vast majority are found in low abundance. Researchers refer to these low-abundant species as the "rare biosphere" of microbiomes[38]. This rarity can be visualized in a rank abundance plot, where the long tail represents these rare species after arranging them from high to low abundance. A challenge in studying this area is the lack of a universal cut-off for defining rarity. Most studies arbitrarily consider species with less than 0.1% abundance as rare or low-abundant. Applying this threshold reveals that over 95% of species in the human gut microbiome fit this category. Despite this prevalence, the majority of microbiome research has concentrated on the functional role of high-abundance species, leaving the role of rare species relatively unexplored.

In this study, we investigated the role of four low-abundant gut bacterial species—*B. paravirosa, C. comes, M. indica,* and *A. butyriciproducens*—in host health. These were chosen for their ability to produce short-chain fatty acids (SCFAs), a trait we focused on due to SCFAs' known importance in gut homeostasis. More specifically, these selected species were found to be low-abundant and possess genes responsible for producing butyrate, a major SCFA with demonstrated positive effects on gut health. Several studies have revealed that butyrate-producing genera such as *Alistipes, Barnesiella, Faecalibacterium, Oscillibacter, Agathobacter,* and *Ruminococcus* are depleted in patients with Inflammatory Bowel Disease (IBD) [39]. Additionally, results from randomized controlled trials have shown that Fecal Microbiota Transplantation (FMT) can replenish lost diversity and ameliorate Ulcerative Colitis (UC) symptoms in affected patients [40, 41]. . However, the efficacy of defined bacteriotherapy or probiotic therapy in treating Crohn's Disease (CD) remains inconclusive [42, 43]. This uncertainty might be attributed to the fact that most interventions have been carried out using high-abundant taxa, such as Bifidobacteria and Lactobacilli [44]. The analysis was conducted using both germ-free and human microbiota-associated mouse models. We formed a synthetic consortium (SC-4) with the selected species and closely monitored their impact on host health, laying the groundwork for potential therapeutic applications targeting low-abundant, SCFA-producing gut bacteria.

Several traits common to SCFA-producing high-abundant species were also observed in the species we examined in our study. Specifically, SC-4 was found to colonize germ-free mice in large numbers and produce SCFAs **(Fig. 2)**. Importantly, the colonized mice displayed no signs of infection or pathology, indicating the safety of the strains. The primary effect of SC-4 colonization in germ-free mice was an increase in the expression of MUC-1 and Muc2 genes and an increase in mucin thickness in the colon. These results bear resemblance to those found in treatment with sodium butyrate, which quadrupled ex vivo mucin synthesis in colonic biopsy specimens [45]. Similarly, treating human-polarized goblet cell lines with butyrate as an energy source augmented the expression of various MUC genes [46]. SC-4 colonization also conferred protection against DSS-induced colitis **(Fig. 3)**. Aware that the protective efficacy observed in a germ-free animal model might not translate to a more complex microbiota environment, we tested SC-4's effectiveness using a human microbiota-associated mouse colitis model. Surprisingly, SC-4 not only cured the mice of colitis but also increased the microbiome diversity to a healthier state **(Fig. 5-6)**. *M. indica,* however, failed to colonize in this intricate setting. We noticed a similar increase in mucin thickness but did not observe any difference in MUC gene expression. Instead, a notable increase in iNos gene expression was detected, reminiscent of iNos-mediated growth in colonic mucus observed in a colitis rat model [47]. We hypothesize that the same mechanism might apply to humanized mice in protection and cure. There were no significant changes in the adaptive immune response in both germ-free and humanized mice. Finally, an examination of patients' fecal metagenome samples

revealed a decrease in the median abundance of all four bacteria in UC and CD patients, although this reduction was not statistically significant.

The inability of the SC-4 to show any visible differences in the adaptive immune profile as well as major signaling pathways compared to DSS treated mice prompted us to ask whether the seen phenotype have any association with functional redundancy maintained by the Humanized mice gut community. The gut community of the humanized mice was stable enough as seen through the replicacy in the bacterial abundance data for biological controls. We also found that the functionality of SCFA production was retained by the community with an increase in some of the low abundant SCFA producers after the DSS treatment **(Fig 5G)**. This is an experimental proof for Insurance hypothesis being in existence for gut microbiome communities similar to what have reported for other biotic communities . This hypothesis extends from a broader theory that biodiversity sustains ecosystem stability by enabling alternative species to take over functional roles if others fail under altered conditions. Applied to the microbiome, this diversity enables the microbial community to adapt to changes like diet alterations, infections, or antibiotic treatments, continuing to provide vital functions such as nutrient assimilation, immune system regulation, and resistance to pathogens[48]. This adaptative quality serves as an "insurance" against disruptions that could lead to dysfunction or illness. The noted phenomenon was vanished when the system is introduced with SC-4. This pose important questions in microbiome research, Does the prevalence studies identifying specific bacterial species presence in different physiological states correspond to this insurance species? Can we categorize a bacterial species to be associated with a disease by mere increase in abundance in gut community during the disease course? These questions have to answered before categorizing any bacteria to be positively associated to a disease. For instance, without considering this hypothesis one can argue that the increased abundant species in this study have positive association to IBD.

Altogether, our findings enhance the fundamental understanding of the functions of low-abundant species and demonstrate a proof-of-principle study for exploring these species as potential probiotic candidates. These results align with the biological insurance hypothesis within gut microbial ecology [49]. Specifically, in the context of Inflammatory Bowel Disease (IBD), low-abundant species we tested may assume the roles of depleted high-abundant taxa such as *Alistipes, Barnesiella, Faecalibacterium, Oscillibacter, Agathobacter, and Ruminococcus* [39]. Further population-level studies will be needed to confirm this possibility.

Materials and Methods

Animal experiments

Six-week-old C57BL/6 Germ-free (GF) mice were procured from Taconic Biosciences Inc., New York, USA and the Mayo Clinic, Rochester, USA. In an effort to negate any bias associated with gender differences, we ensured the inclusion of both male and female mice in our experiments, where feasible. All animals were subject to a 12-hour light/dark cycle and provided unlimited access to sterile drinking water and a standard chow diet. The protocols for all animal experiments were reviewed and duly approved by the South Dakota State University (SDSU) Institutional Animal Care and Use Committee (Approval #19-014A).

Bacteria culture and maintenance

The bacterial species utilized in our experiments were cultured and maintained using DSMZ's modified PYG media, supplemented with L-cysteine as a reducing agent and resazurin as an oxygen indicator. Cultures were preserved at 37°C within an anaerobic chamber (Coy Lab Products Inc., MI, USA), filled with an atmospheric composition of 85% nitrogen, 10% carbon dioxide, and 5% hydrogen.

For *in-vitro* phenotypic assessment of butyrate production, overnight bacterial cultures were prepared under three distinct media conditions. These included Brain Heart Infusion broth and yeast extract-based media, both with and without inulin as a complex carbohydrate supplement. The third medium utilized was DSMZ's modified PYG medium. Following 24 hours of incubation in the anaerobic chamber, 100µL of the culture was combined with 500µL of 5% freshly prepared meta-phosphoric acid. This mixture was then thoroughly vortexed for two minutes and promptly stored at -80°C for subsequent gas chromatography (GC) analysisFor inoculum preparation, the optical density (OD_{600}) of overnight grown cultures was adjusted to one, and then aliquots were stored in 12% DMSO at -80°C until further use. On the day of inoculation, an equal volume of all four bacteria were resuspended and washed twice in PYG media. After centrifugation at 7000 RPM for 10 minutes, the bacterial pellets were reconstituted in PYG media to get a 20x concentrated bacterial suspension. 100uL / mice of bacterial suspension were prepared and transferred to GF mice units in sterile, airtight 2mL glass vials.

Mice experiments

In our experiments involving gnotobiotic mice, we utilized a mixture of four butyrate-producing bacterial strains, referred to as the ‘Short-chain fatty acid producing consortium” (SC-4), for inoculation. A 200µL dose of this 20X concentrated bacterial mixture was administered to the treatment group via oral gavage. Each experimental group consisted of 4-6 mice, with an additional group of uninoculated mice serving as the germ-free control. Each mouse received two inoculations (one per day) and was subsequently allowed a colonization period ranging from a minimum of 14 days to a maximum of 28 days. At the conclusion of the colonization period, all mice were euthanized via cervical dislocation, and samples were collected for subsequent analysis

Colitis was chemically induced in germ-free (GF) mice following the protocol described by Wirtz et al. [50], with slight modifications. Dextran sulfate sodium salt (30-50 KDa) from MP Biomedicals was included at a concentration of 1.5% in autoclaved drinking water. Mice had ad libitum access to this DSS-infused water for six days, after which they were euthanized via cervical dislocation, with samples subsequently collected for further analysis. Beginning on the fifth day, all animals started displaying signs of rectal bleeding. One mouse from the GF group treated with DSS died on the sixth day of treatment. The onset of colitis was validated through a fecal occult test conducted on the third day.

In the case of humanized Fecal Microbiota Transplant (hFMT) mice, both male and female six-week-old mice received oral gavage of 200µL of the pooled fecal sample over three consecutive days, as prepared in our previous work [33]. After several generations of breeding, male and female mice were exposed to 2.5% Dextran Sulfate Sodium (DSS) in their drinking water for five days to induce colitis. The mice then switched to regular drinking water for another three days before being sacrificed for the DSS group. For the control group, mice were allowed to naturally recover for the subsequent 23 days. For the BP and IBP groups, mice were gavaged with 200µL of a 20X concentrated bacterial mixture for two days, or the bacterial mix along with 1% inulin in their drinking water. In the case of the EBP, EIBP, and EI groups, humanized mice were treated with the

bacterial mix, the bacterial mix along with inulin, or inulin only, and were then sacrificed after 23 days. Cecal and colon fecal samples were collected for 16S amplicon sequencing.

Fecal occult blood test

The initiation of colitis was confirmed via an occult blood test utilizing fecal samples. For this test, Whatman filter paper pre-treated with guaiac was employed. A thin smear of feces was applied to one side of this guaiac-coated Whatman filter paper. Subsequently, one or two drops of 3% hydrogen peroxide were carefully administered to the opposite side. A rapid transition to a blue color on the Whatman paper was interpreted as a positive indication of blood presence in the sample

Bacterial enumeration

Total bacterial count in the fecal samples collected at different time points was assessed to evaluate bacterial colonization. Anaerobic modified PYG media (DSMZ medium) was used to enumerate the bacterial load as this media was found to be supportive for all four bacteria used in this study.

Genomic DNA extraction and targeted amplicon sequencing

Extraction of genomic DNA from pure bacterial culture was performed using DNeasy blood and tissue kit. We used the DNeasy power soil kit (Qiagen, Maryland), to extract from the fecal samples and intestinal contents of the mice, as per the manufacturer's instructions. The 16s rRNA gene sequencing was performed according to standard Illumina protocol, where PCR amplicons targeting the V3-V4 region of the bacterial 16s rRNA gene were used for sequencing. Locus-specific primers primer pairs (16S Amplicon PCR Forward Primer = 5' CCTACGGGNGGCWGCAG, and 16S Amplicon PCR Reverse Primer = 5' GACTACHVGGGTATCTAATCC) are attached with overhang adaptors (Forward overhang: 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG, and Reverse overhang: 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG) at the 5' end of the respective primer sequences (Illumina, inc.) and used for amplifying the region of interest.

The resulting amplicons thus generated were then cleaned from free primers and primer dimer species using AMPure XP beads (Beckman Coulter). Sequencing libraries were prepared using Nextera XT library preparation kit (Illumina, Inc.). After indexing using a dual barcoding system per the manufacturer's protocol and then normalized to a concentration of 4nM, the libraries were pooled into a single loading library. Sequencing was performed on the Illumina Miseq platform using 2x300 bp paired-end read chemistry. Demultiplexed and adaptor-trimmed reads generated by Illumina analytical tools were then used for further processing.

Bacterial community profiling

Microbiota profiling from 16S sequencing was performed using Vsearch [51]. Merging and Quality filtering with a minimum cutoff length of 400 and max length of 500 were done for the fastq files using Vsearch tool. Singleton and chimeric reads (UCHIME) were removed. OTU picking was performed with VSEARCH abundance-based greedy clustering. OTUs were annotated with the SILVA reference database [52] or Custom database made from Full-length 16s extracted from the whole genome of the four bacteria in the consortia. For the creation of the custom database for SCFA producing bacterial consortium we took the full length 16s rRNA sequence from SILVA/NCBI. The resulting OTU table is further processed for analysis. Alpha and Beta diversity calculation and plotting were done using Microbiome analyst with default parameters [53].

Bacterial species abundance mapping

To assess the association of selected four butyrate producers in IBD conditions, an abundance mapping at the strain level was performed using publicly available 1338 gut metagenome sequencing data sets collected from US individuals as a part of a longitudinal cohort research study by Lloyd et al[54]. For all the metagenome reads quality trimming and adapter clipping were done with Trimmomatic [55]. Further, the reads were aligned against the human genome to filter out human reads and were assembled with Bowtie2 v2.3.2 [56] and SAMtools [57]. The resulting contigs were classified taxonomically by k-mer analysis using Kraken2 [58], with Kraken 2 standard bacterial database built using Kraken-built. Subsequent estimation of species abundance was performed using Kraken-tools [59].

Gas chromatography (GC) analysis for SCFAs

Immediately before GC analysis, samples were thawed and vortexed for 2 minutes. The ethyl acetate (EA) extraction method described by Garcia-Villalba et al [60] was used to detect SCFA. In brief, the homogenized sample was then centrifuged for 10 min at 17,949 × g. Each milliliter of supernatant was extracted with 1 mL of organic solvent (EA) for 2 minutes and centrifuged for 10 minutes at 17,949 × g. A minimum of 200uL organic phase volume per sample was used to load in the Trace 1300 gas chromatogram (Thermo fisher scientific).

Quantitative RT-PCR-based Gene expression profiling

To assess the localized response of host-microbe interaction following colonization of BP4 strains, 100mg of tissue from the colon was sampled and snap-frozen in liquid nitrogen. The samples were then saved at -80oC until further processing. For RNA extraction, we used a TRIzol® (Ambion, life technologies)- chloroform (Sigma-Aldrich) method. According to the manufacturer's protocol, a DNase treatment was performed using RNase-Free DNase kit (Qiagen, Maryland) to remove any contaminant genomic DNA. The RNA quality and quantity were evaluated using NanoDrop One (Thermo Scientific) and saved at -80°C until further use.

Complimentary DNA (cDNA) was prepared from 250 ng of total RNA using the First strand cDNA synthesis kit (New England BioLabs Inc.) as per the manufacturer's protocol. Host gene expressions were assessed by qRT-PCR using the Rt2 profiler array (Qiagen, Maryland) **(Supplementary. Table1)** or 25 define immune-related genes **(Supplementary. Table2)**. Reactions were prepared using Power SYBR® Green PCR Master mix (Applied Biosystems). PCR run was performed in an ABI7500 standard (Applied Biosystems) RT-PCR machine using the following cycling conditions: 95°C for 10 mins, 40 cycles of 95°C for 15 sec and 60°C for 1 min.

Raw cycle threshold (C_T) values at a threshold of 0.15 were exported and then uploaded to the Qiagen data analysis center for further analysis. A C_T cut-off value of 30 was set for analysis. An average geometric mean of C_T values of two housekeeping genes - mouse beta-actin (Actb) and mouse glyceraldehyde phosphate dehydrogenase (Gapdh) was used for data normalization and ΔC_T calculation. Fold change and fold regulation in the gene expression were calculated using the $\Delta\Delta C_T$ method.

Separation of lymphocytes from Peripheral mouse blood

Immediately after euthanasia, blood was collected via cardiac puncture, transferred to heparinized blood collection vials (BD Vacutainer), and gently mixed to avoid coagulation. SepMate TM PBMC

isolation tubes (STEMCELL Technologies) were used to collect lymphocytes from the blood. An equal volume of DPBS (Dulbecco's PBS) was added to the blood sample before transferring to the SepMate TM tubes prefilled with 4.5 mL of Lymphoprep (STEMCELL Technologies) density gradient medium. Tubes were centrifuged at 1200 x g for 10 minutes at room temperature. The top layer containing the plasma and the mononuclear cells (MNC) was transferred to a fresh 15mL falcon tube by a single pour-off step. After washing two times with DPBS, transferred MNCs were re-suspended in freezing media containing 10% DMSO and stored in liquid nitrogen until staining for flow cytometry.

Separation of lymphocytes from mouse spleen

Spleen was mechanically disrupted using sterile BP blades after removing the capsular layer in a sterile Petri dish. The cell suspension in RPMI1640 media (Corning) was filtered through a cell strainer (70uM Nylon mesh, Fisherbrand) to make a single cell suspension. Cells were washed in RPMI 1640 (Corning) once and then treated with ammonium chloride solution (STEMCELL Technologies) at a 9 :1 ratio with RPMI 1640 cell culture media for 7 minutes on ice to lyse RBCs. After lysing RBCs, the cells were washed twice with RPMI 1640 media, resuspended in freezing media containing 10% DMSO, and stored in liquid nitrogen until further use.

Flow cytometry analysis

On average, $10^5 – 10^7$ cells were used for staining. A list of antibodies with fluorochrome is used as in **(Supplementary. Table3,4)**. Stained cells were fixed in IC fixation buffer (Thermofisher) and run in Attune NxT Flowcytometer (Thermofisher). Raw files were exported to FlowJo software for further analysis.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). Comparisons between groups were analyzed using either the Mann-Whitney t-test or the t-test with Welch's correction. A p-value of < 0.05 was considered statistically significant. Bubble plot was plot in R using ggplot package.

Data Availability Statement

All 16s amplicon sequencing data generated from this project have been deposited in the NCBI SRA database under BioProject PRJNA1013066. Raw whole-genome sequence data for the strains used is from previously published study [27] under the BioProject PRJNA494608.

Ethics Statement

All the animal procedures were approved by the Institutional Animal Care and Use Committee of South Dakota State University with the Prior approval of protocols 19-014A

Author Contributions

Funding

Conflict of Interest

Acknowledgments

Figures

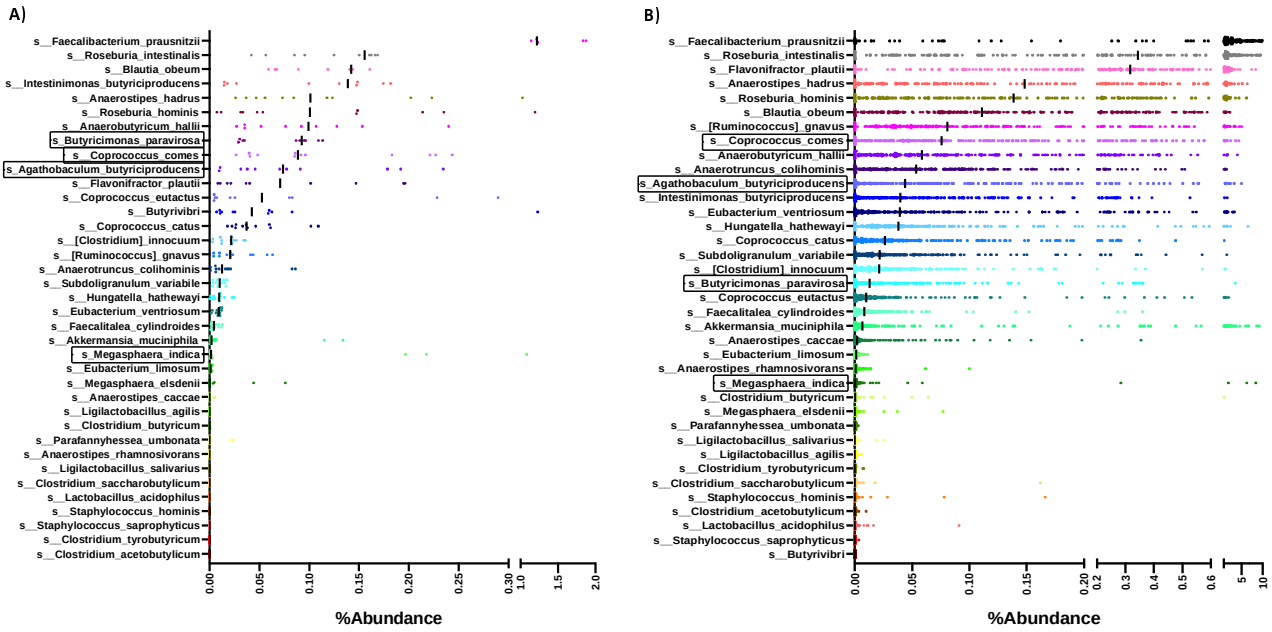

Figure 1. Butyrate-synthesizing bacteria majorly belong to the low dominant category of the human gut. Percentage abundance of Butyrate-producing bacteria in Donor **(A)** and healthy people fecal metagenome samples from a previously published study [61] **(B)**. The box is the bacteria present in the SC-4 consortia.

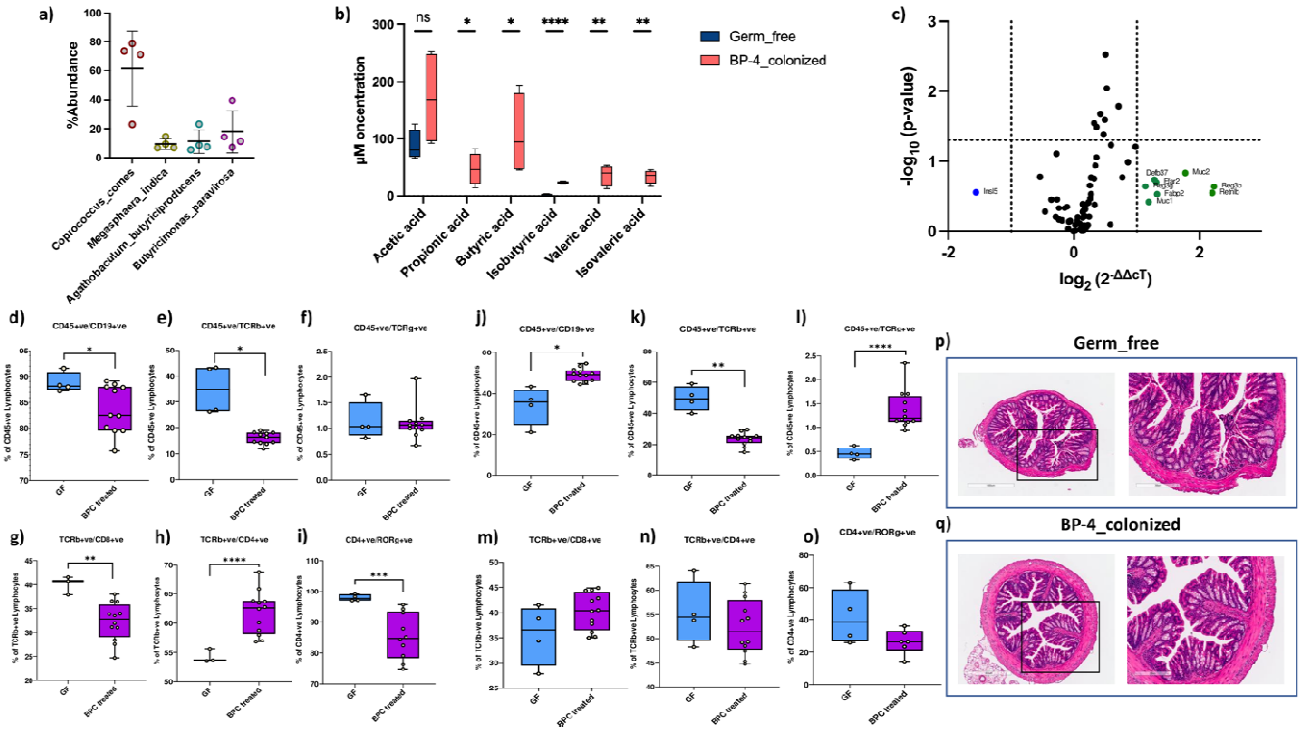

Figure 2. SC-4 got successfully colonized in GF mice and increase mucin thickness. Percentage abundance of SC-4 bacteria **(A)** and concentration of SCFAs in mice cecum **(B)** colonized with SC-4. RT-PCR gene expression for Rt2 profiler array consisting of 88 genes of mice colon, colonized with SC-4. Green dots - overexpressed more than 2-fold change, Blue dots- represses more than 2 fold compared to GF control (dotted lines on the x-axis represent a 2-fold increase or decrease in expression and the y-axis represents a p-value cutoff of 0.05) **(C)**. Percentage cell population of lymphocytes in the spleen of GF and GF colonized with SC-4 mice **(D-I)**. Percentage cell population of lymphocytes in the spleen of GF and GF colonized with SC-4 mice **(J-O)** Representative histopathological photograph showing the colon tissue cross-section after H&E staining GF mice **(P)** GF colonized with SC-4 **(Q).**

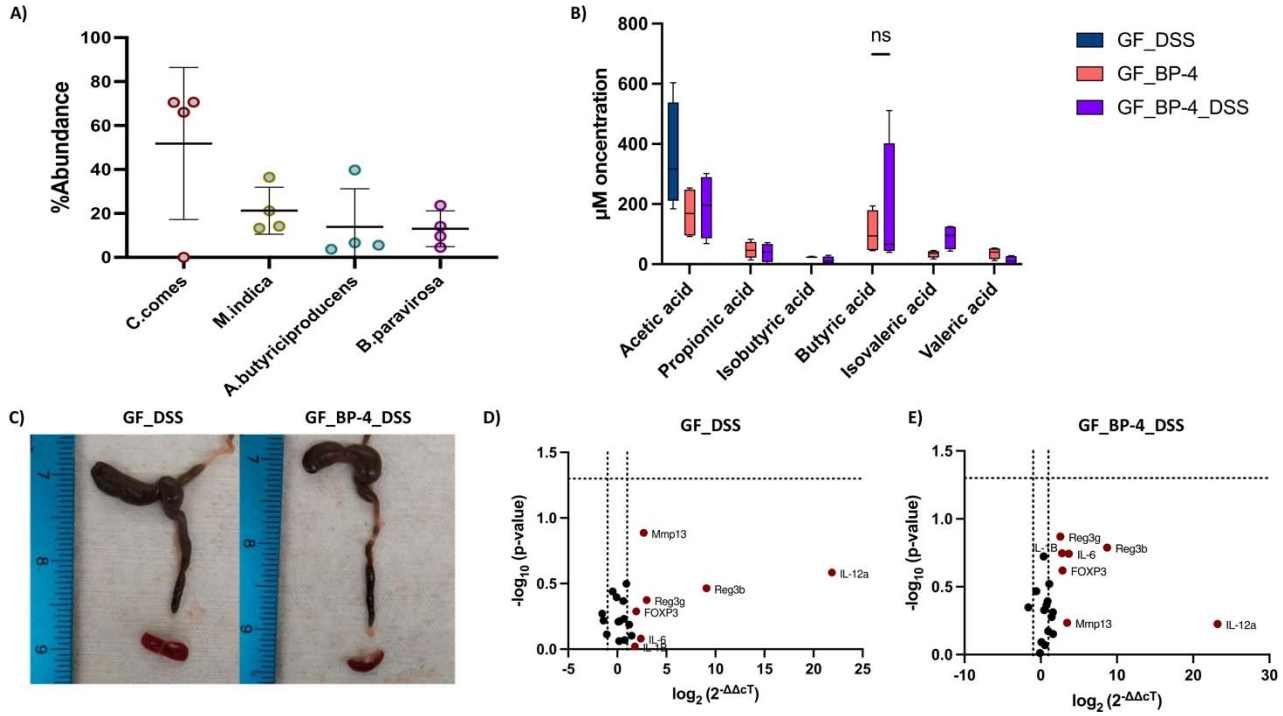

Figure 3. SC-4 confers protection against DSS-induced colitis. Percentage abundance of SC-4 bacteria **(A)** and concentration of SCFAs **(B)** in mice cecum upon DSS treatment. Representative colon from GF mice and SC-4 pretreated mice induced with colitis **(C)**. RT-PCR gene expression for selected 25 immune-related genes of mice colon, mice either induced with colitis **(D)** or pre colonized with SC-4 and then induced with colitis **(E)**. Green dots - overexpressed more than 2-fold change, Red dots- overexpresses more than 2 fold compared to GF control (dotted lines on the x-axis represent a 2-fold increase or decrease in expression and the y-axis represents a p-value cutoff of 0.05).

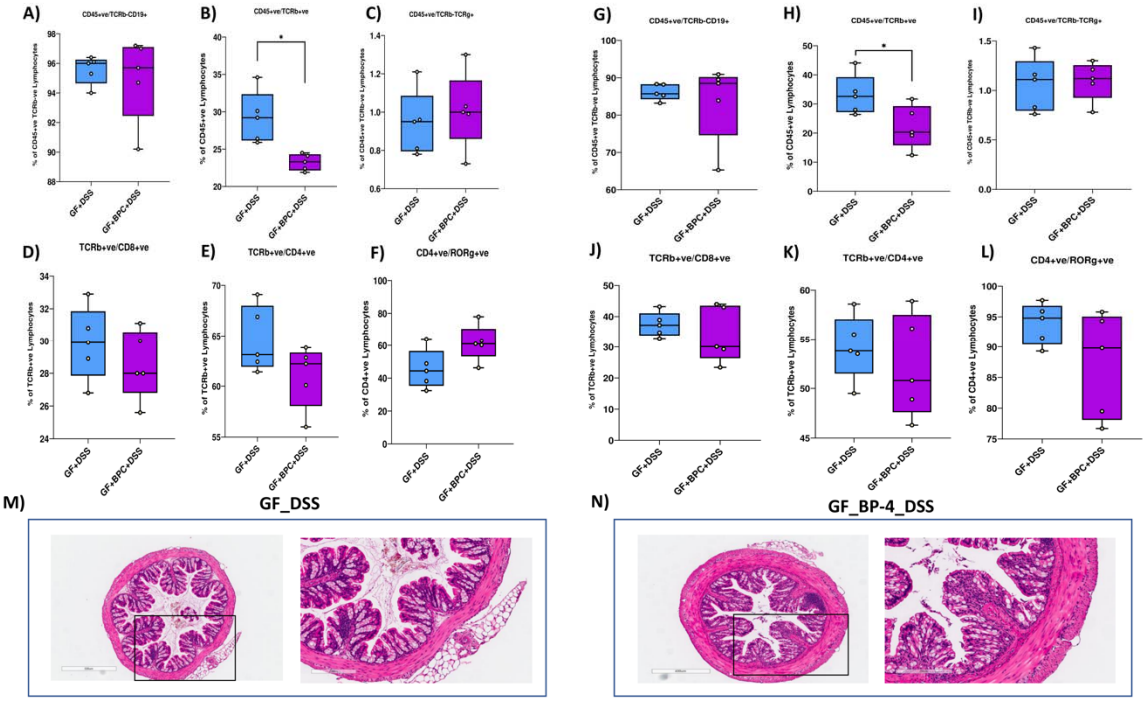

Figure 4. SC-4 confers protection against colitis by increasing the mucin thickness. The percentage cell population of lymphocytes in the spleen of GF and GF colonized with SC-4 induced with colitis **(A-F)**. The percentage cell population of lymphocytes in the spleen of GF and GF colonized with SC-4 mice induced with colitis **(G-I)** Representative histopathological photograph showing the colon tissue cross-section after H&E staining GF mice **(P)** and GF colonized with SC-4 **(Q)** induced with colitis**.**

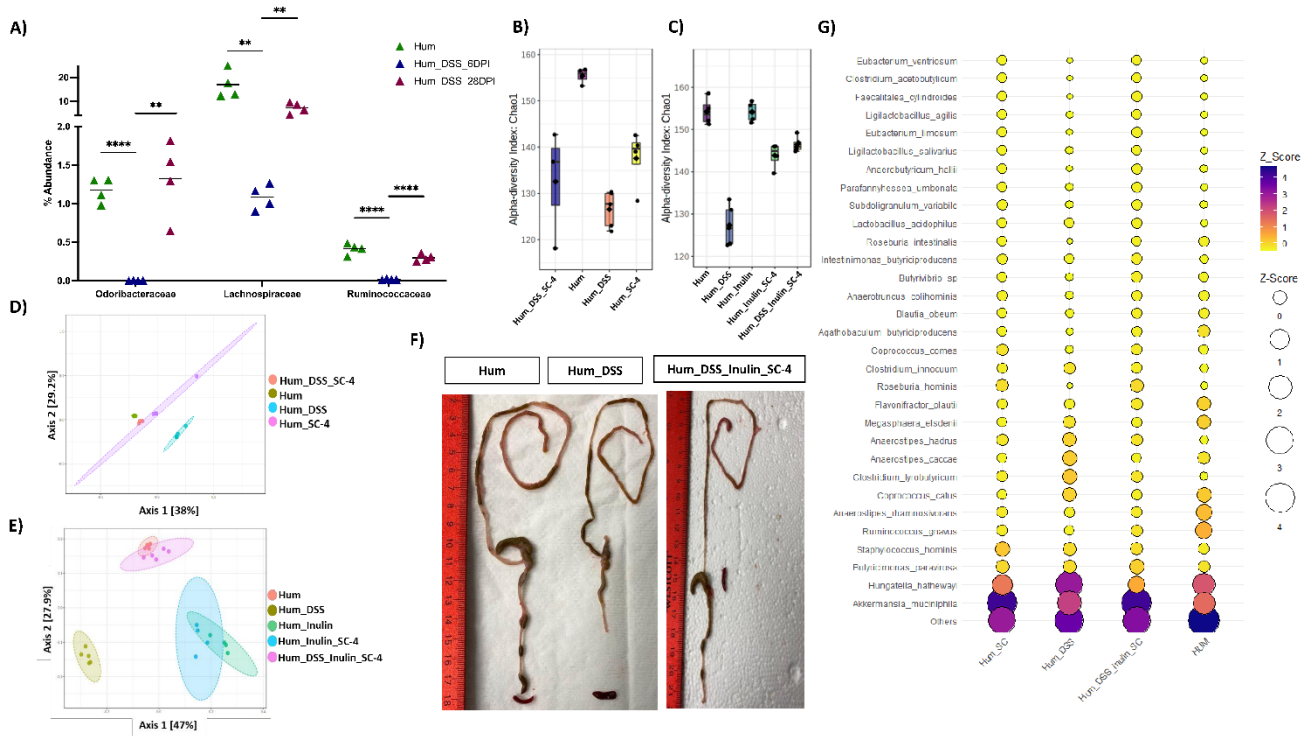

Figure 5. BP- 4 colonization increases Microbiome diversity. Percentage abundance of *Butyricimonas paravirosa* (Odoribacteraceae), *Coprococcus comes* (Lachnospiraceae), or *Agathobaculum butyriciproducens* (Ruminococcaceae) in Humanized mice induced with colitis at 6th and 28th Day post-infection **(A)**. Alpha diversity analysis (Chao1) when mice with DSS-induce colitis treated with SC-4 **(B)**. and treated with SC-4 with supplementation of Inulin in mice diet **(C)**. Beta diversity analysis (Chao1) when mice treated with SC-4 **(D)** and when mice treated with SC-4 and supplemented with Inulin in mice diet **(E)**. Representative colon from humanized mice (control), Humanized mice with DSS-induce colitis (DSS), and humanized mice with DSS-induce colitis and treated with SC-4 and supplemented with Inulin (IBP) **(F)**. Relative abundance Z-score at a species level resolution for SCFA producing bacteria (Similar to Figure 1) identified in the DSS induced humanized mice feces with and without SC-4/Inulin administration (g). (**Labels** - BP4 – SC-4 treated humanized mice after colitis induction, Control – Humanized mice without any treatment, EBP - SC-4 treated humanized mice, CRN – colitis recovered naturally, Control – Humanized mice without any treatment, CRN – colitis recovered naturally, EIBP - SC-4 treated humanized mice supplemented with Inulin in mice diet, EI – Humanized mice supplemented with Inulin only, IBP - SC-4 treated humanized mice supplemented with Inulin in mice diet after colitis induction)

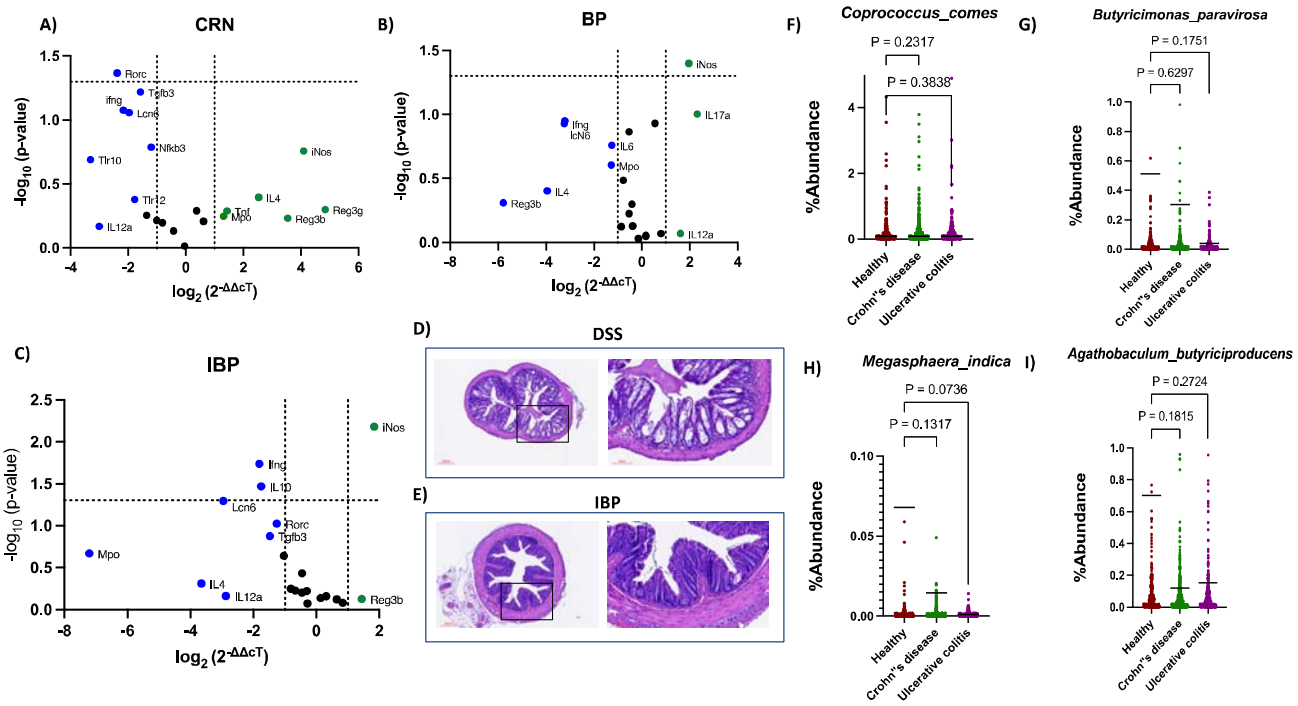

Figure 6. SC-4 colonization in Humanized mice increases iNOS gene expression. RT-PCR gene expression for selected 25 immune-related genes of mice colon, mice either induced with colitis recovered naturally **(A)** or induced with colitis and treated with SC-4 **(B)** finally mice induced with colitis and treated with SC-4 and supplemented with Inulin**(C)**. Green dots - overexpressed more than 2-fold change, Green dots- overexpresses more than 2-fold compared to GF control (dotted lines on the x-axis represent a 2-fold increase or decrease in expression and the y-axis represents a p-value cutoff of 0.05). Representative histopathological photograph showing the colon tissue cross-section after H&E staining humanized mice induced with colitis **(D)** humanized mice induced with colitis and then treated with SC-4 along with Inulin supplementation **(E)**. The abundance of *Coprococcus comes* **(F)** *Butyricimonas paravirosa* **(G)** *Megasphaera indica* **(H)** *Agathobaculum butyriciproducens* **(I)** in UC, CD patient, and healthy people sample from a previously published study[28].

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