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Bacterial Swarmers Enriched During Intestinal Stress Ameliorate Damage

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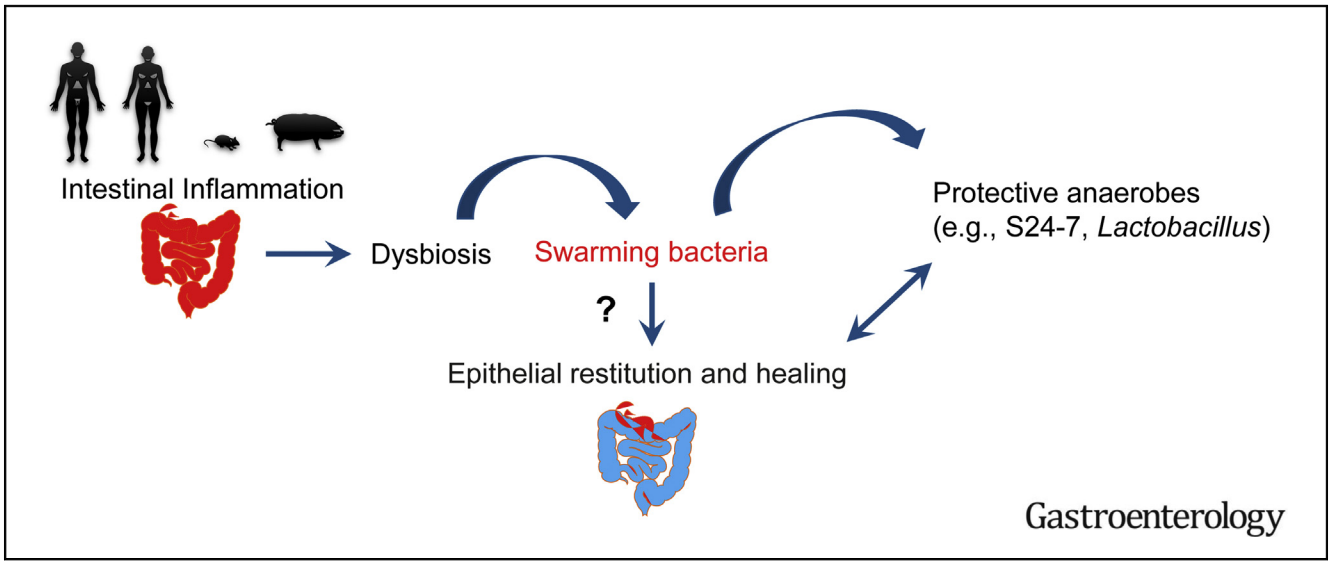

BACKGROUND AND AIMS: Bacterial swarming, a collective movement on a surface, has rarely been associated with human pathophysiology. This study aims to define a role for bacterial swarmers in amelioration of intestinal stress. **METHODS:** We developed a polymicrobial plate agar assay to detect swarming and screened mice and humans with intestinal stress and inflammation. From chemically induced colitis in mice, as well as humans with inflammatory bowel disease, we developed techniques to isolate the dominant swarmers. We developed swarm-deficient but growth and swim-competent mutant bacteria as isogenic controls. We performed bacterial reinoculation studies in mice with colitis, fecal 16S, and meta-transcriptomic analyses, as well as in vitro microbial interaction studies. **RESULTS:** We show that bacterial swarmers are highly predictive of intestinal stress in mice and humans. We isolated a novel *Enterobacter* swarming strain, SM3, from mouse feces. SM3 and other known commensal swarmers, in contrast to their mutant strains, abrogated intestinal inflammation in mice. Treatment of colitic mice with SM3, but not its mutants, enriched beneficial fecal anaerobes belonging to the family of Bacteroidales S24-7. We observed SM3 swarming associated pathways in the in vivo fecal meta-transcriptomes. In vitro growth of S24-7 was enriched in presence of SM3 or its mutants; however, because SM3, but not mutants, induced S24-7 in vivo, we concluded that swarming plays an essential role in disseminating SM3 in vivo. **CONCLUSIONS:** Overall, our work identified a new but counterintuitive paradigm in which intestinal stress allows for the emergence of swarming bacteria; however, these bacteria act to heal intestinal inflammation.

Keywords: Intestinal Stress; Protection; Feces; Enterobacter; S24-7.

Bacterial motility is essential in mucosal colonization and has long been associated with virulence and pathogenesis.[1,2] Intestinal inflammation, such as inflammatory bowel disease (IBD), is attributed to dysbiosis and the mucosal immune system.[3] The disease is characterized by enrichment of motile flagellated bacteria resident in the microbiome and its encroachment into the inner mucus layer and the intestinal epithelial cells.[4–6] However, despite cues of the molecular mechanisms of flagella during intestinal health and disease,[7–11] the functional importance and consequence of bacterial motility in a microbial consortium is unknown.

Swimming and swarming are the two primary and common forms of bacterial motility.[12] Swarming, driven by flagella, is a distinct process in certain groups of bacteria characterized by collective and rapid movement across a surface.[12,13] This process, in contrast with swimming in liquid, offers bacteria a competitive advantage in occupying specific niches (eg, seeding colonization);[14] however, the cost-benefits to bacteria[15,16] and consequences to its host or the environment remain primarily unknown.[17]

We hypothesized that bacterial swarming is a necessary adaptation to a noxious environment in a host such as bacteria within inflamed or stressed intestines. Because prototypical swarming bacteria (eg, *Proteus mirabilis* and *Pseudomonas aeruginosa*) are associated with virulence,[17,18] we surmised that bacterial swarming might be well represented in colonoscopy aspirates from humans with bacterial virulence-associated pathologies (eg, intestinal inflammation).[19] This study aims to determine the occurrence and consequence of bacterial swarming in humans and in the animal kingdom, in the context of a stressed and non-stressed intestinal environment. In addition, we aim to uncover potential mechanisms by which swarming bacteria interact with the host.

WHAT YOU NEED TO KNOW

BACKGROUND AND CONTEXT

Bacterial swarming is defined as collective movement of cells on a surface. As opposed to biofilms, bacterial swarming has rarely been associated with host pathophysiology.

NEW FINDINGS

Presence of bacterial swarmers is a feature of a stressed intestine. Bacterial commensal swarmers can protect from intestinal inflammation, when present in high abundance, in a microbiome-dependent manner. A novel swarming bacterium *Enterobacter* species SM3 can enrich the S24-7 group of bacteria, associated with inflammatory bowel disease remission.

LIMITATIONS

This study lacks direct evidence of in vivo bacterial swarming.

IMPACT

This study encourages isolation and banking of bacterial swarmers as a potential personalized probiotic approach.

Materials and Methods

Isolation of Bacterial Swarmers From Feces and Colonoscopic Aspirates

Patients diagnosed with either IBD (Crohn's disease or ulcerative colitis) or undergoing routine screening colonoscopy for colorectal polyps/cancer or who required a colonoscopy as part of their medical management of any gastrointestinal disorder as clinically indicated were recruited for the study. Sixty-three patients consented to participate in a colonoscopy aspirate or fecal collection study, which was approved by the Institutional Review Board (#2015-4465; #2009-446; #2007-554). Bacterial swarmers were isolated on Luria Bertani (LB) swarming agar medium containing 5 g/L agar with some modifications to an established method.[20] To isolate a singular dominant swarmer from a polymicrobial mix of bacteria (such as feces), we initially focused on developing an assay to isolate swarmers using known polymicrobial mixed cultures of bacteria. Single bacterial species (up to 7 strains belonging to different taxa) grown in LB (optical density measured at a wavelength of 600 nm [OD_{600}] reached 1.0–1.3) were mixed in a 1:1 ratio and, 5 μL of this mix was spotted on 0.5% agar plates. After air drying at room temperature, the plates were incubated at 37°C, 40% relative humidity for 10 hours. Bacterial swarm front was swabbed using a sterile toothpick from the edge of swarming colony at different locations (see *arrows*, Supplementary Figure 1) and after restreaking on separate agar plates and scaled by growth in LB, the bacteria in the samples were identified using Matrix Assisted Laser Desorption and Ionization–Time of Flight (MALDI-TOF). Swarmers present in the fecal or colonoscopic samples were isolated and determined using an identical approach. Fecal pellets and/or colonoscopy aspirates from the clinic and/or feces of mice and pigs were collected in sterile tubes and were freshly homogenized in phosphate-buffered saline (PBS) for swarming assays. Most bacterial swarmers were detected within the first 48–72 hours from incubation. Dominant swarmers from the edge of the colony were identified using MALDI-TOF. Once identified, cells from the same aliquot were plated on to 1.5% LB agar and serially passaged from a single colony to obtain a pure culture of the strain. Details of the procedure are presented in Supplementary Materials.

* **Authors share co-first authorship.**

Abbreviations used in this paper: **bp, base pair; CI, confidence interval; DSS, dextran sulfate sodium; GF, germ free; IBD, inflammatory bowel disease; LB, Luria Bertani; MALDI-TOF, Matrix Assisted Laser Desorption and Ionization–Time of Flight; OD, optical density; OTU, operational taxonomic units; qPCR, quantitative polymerase chain reaction; rRNA, ribosomal RNA; SPF, specific pathogen-free; WT, wild-type.**

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Characterization of the Bacterial Strains

Swarming ability of a single bacterial species using a pure culture of *Enterobacter* species SM1 and its isogenic mutant, *Enterobacter* species SM3 and its transposon mutants, *Serratia marcescens* Db10 and JESM267, clinical isolate of *Serratia marcescens*, *Bacillus subtilis* 3610 and its isogenic mutant DS215 was always determined on LB swarming agar at 37°C and 40% relative humidity prior to any experiments using these strains. *B subtilis* 3610 and its isogenic mutant were compared on LB swarming agar containing 0.7% agar.[21] To capture real-time swarming motility, a temperature- and humidity-controlled incubator equipped with time lapse photography was built and swarming area was calculated using a python-based script (Nature Protocol Exchange for detailed protocol, https://doi.org/10.21203/rs.2.9946/v1). Growth kinetics was observed in LB broth, whereas swimming potential of the strains was assessed in freshly grown cultures (OD_{600} ~ 0.3) or 0.3% LB agar. Surfactin synthesis was determined using blood agar hemolysis,[22] drop-collapse,[23] and drop-counting assay.[24] Swarming on mucosal surface was demonstrated using a colon tissue from mice that was treated with 3% dextran sulfate sodium (DSS) via a mucosal race experiment. Details of the techniques are presented in Supplementary Materials.

Mouse Model Studies

Four- to six-week-old female C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine; # 000664) were co-housed for acclimatization at the vivarium for 2 weeks prior to randomization by coin toss as previously described.[25] Five-week-old germ-free (GF) wild-type (WT) C57BL/6 mice were transferred to specific pathogen-free (SPF) conditions[26] during experimentation (GF/SPF). Acute colitis was induced by administering 3% (wt/vol) DSS (MP Biomedicals, catalog number 160110). To determine the effect of swarming and swarming-deficient strains during colitis, WT mice were orally gavaged with 100 μL (~4×10^9 colony forming unit per milliliter [CFU/mL]) test bacteria or LB as vehicle, daily for 9–12 days until the weight of vehicle group decreased >20%. Swarming-deficient strains were generated either using recombineering and polymerase chain reaction (PCR) ligation mutagenesis approach[27,28] or mariner-based transposon mutagenesis.[29] GF/SPF mice were gavaged with SM3 or LB and treated for 7 days, when most mice had >10% weight decrease. Daily gavage of bacterial strains required use of unwashed bacterial strains grown in fresh LB (OD_{600} ~ 1.0). To determine the healing effect of SM3 in colitis, C57BL/6 mice were administered 3% DSS in drinking water for 7 days (when most mice had a weight loss >10% of their pre-DSS exposure weight). Subsequently, mice received animal facility drinking water without DSS and were further randomized using coin toss to a treatment group that was delivered 4×10^9 colony forming unit per milliliter (CFU/mL) of bacterial cells or LB by oral gavage for 5 days. At the end of the experiment, mice were humanely killed using isoflurane anesthesia or CO_2 asphyxiation and intestines harvested for hematoxylin-eosin staining and histopathology. Lipocalin (LCN2) assay was performed using Mouse Lipocalin-2/NGAL Duoset ELISA kit (R&D System, Inc, Minneapolis, Minnesota; Cat. no. DY1857).

The role of TLR5 was assessed in a chronic colitis model of TLR5KO mice administered anti-interleukin10R monoclonal antibody.[30] Mice were orally gavaged with SM1 or SM3 every third day from Day 1 onward. Histology scoring for inflammatory damage was performed according to published criteria for colonic inflammation as a consequence of cytokine imbalance.[4]

Fecal Microbiome Profiling

16S ribosomal RNA (rRNA) meta-analyses of the fecal samples from mice were conducted at Wright Labs, LLC. DNA was isolated from feces using a Qiagen DNeasy Powersoil DNA Isolation kit following the manufacturer's instructions (Qiagen, Frederick, Maryland). The 16S rRNA gene was amplified using Illumina iTag PCR,[31] pooled, gel purified at ~400 base pairs (bp), and multiplexed with other pure libraries to form a sequencing library normalized to the final concentration of library observed within each sample. The sequencing library was sequenced using an Illumina MiSeq V2 500 cycle kit cassette with 16S rRNA library sequencing primers set for 250 bp paired-end reads at Laragen Inc (Culver City, California). The paired-end sequences were merged with a minimum overlap of 200 bases, trimmed at a length of 251 bp, and quality filtered at an expected error of less than 0.5% using USEARCH,[32] analyzed using the QIIME 1.9.1,[33,34] and assigned operational taxonomic units (OTU) using UPARSE at 97% identity.[35] The taxonomy was assigned using the Greengenes 16S rRNA gene database (13.5 release).[36]

In Vitro Coculture Assay Using Muribaculum intestinale

A broth-based or swarm plate–based coculture assay was designed to identify possible interaction between SM3 and *Muribaculum intestinale* (DSM 28989). Early exponential phase cells (OD_{600} 0.5–0.6) grown in chopped meat carbohydrate broth PR II (Becton, Dickinson and Company, Franklin Lakes, NJ) in an anaerobic chamber at 37°C (O_2 = 2%–3%) were used to establish the assay. For broth-based assay, *M intestinale* was grown with fresh cells of SM3/SM3_18/SM1 in a Hungate tube and cells were collected at different time points (21/24, 36, 45/48 hours) for DNA extraction. For swarm plate–based assay, *M intestinale* grown in chopped meat carbohydrate broth PR II was transferred into a bore-well at the center of a swarming plate on which SM3/SM3_18/SM1 swarmed. Plates were incubated at different conditions for 64 hours (aerobic, sealed, or anaerobic) at 37°C. For sealed condition, plates were taped carefully using parafilm to maintain anaerobiosis throughout the experiment. For divided/sealed condition, a small Petri dish was placed inside a big Petri dish, both containing swarming agar. The bore-well containing *M intestinale* was stationed in the small Petri dish, whereas the swarming or less swarming strains were spotted on agar present in the big Petri dish. This allowed physical separation of *M intestinale* from the swarming bacteria, nevertheless maintaining an anaerobic condition in this sealed system. DNA was extracted and quantitative polymerase chain reaction (qPCR) analysis was performed using equal volume of each diluted DNA sample and *M intestinale*–specific primers (Supplementary Table 1). Details of the technique are presented in Supplementary Materials.

Statistical Analysis

P values for statistical tests involving the experimental data were obtained by using appropriate parametric or nonparametric methods, as indicated in the figure legends; 95% confidence intervals (CI) were obtained for the relevant parameters. Normality (Gaussian distribution) for the data was not assumed, to begin with. For each dataset, normality was

tested. If there was not much evidence in favor of normality, then suitable transformations (eg, log normal transformation) were considered to discern whether the transformed data fit a Gaussian distribution. All statistical tests, except where otherwise indicated, were performed with Graph Pad Prism v.8.2.0. All plots depict the mean and 95% CI (except where otherwise indicated) for the relevant parameters.

Results

Presence of Bacterial Swarmers is a Feature of a Stressed Intestine

To test the relationship between fecal abundance of bacteria with swarming potential and human health, we developed a fecal agar-based modified polymicrobial swarming assay.[20] We obtained colonoscopy aspirates from individuals with a progressive illness (IBD – Crohn's and ulcerative colitis and other common forms of intestinal stress like intestinal polyps,[37,38] as well as age- and gender-matched controls [those without a clinically active illness]). Within our sampling pool, bacterial collective spreading on soft agar was over-represented in cases with overt or clinically active intestinal stress (Figure 1*A–B*). As a preliminary assessment, we judged bacterial swarmers' presence in feces by the bacterial spread with a surfactant layer on soft agar. Swarmers were isolated, identified using MALDI-TOF, and validated for their swarming motility (Table 1).

In this pilot evaluation, the specificity and positive predictive value of the test for disease as defined was approximately 88% and 89%, respectively. In comparison, the test's sensitivity and the negative predictive value was only approximately 56% and 52%, respectively (Figure 1*C*). Similarly, feces collected from a limited sample size of pigs with active IBD also showed an increased qualitative trend of collective spreading and swarming compared with control pigs (Figure 1*D*).

Novel Enterobacter *Swarming Strains Were Isolated From Mouse Feces*

Next, we focused on isolating endogenous swarming bacteria residing in rodents and humans. An initial approach was to determine if a single dominant swarming species could always be isolated from a polymicrobial culture (eg, mammalian feces). In our competitive swarming assay, a mix of different pure bacterial cultures gave rise to a single bacterial species populating the leading edge of the swarm colony on agar (Supplementary Figure 1*A–B*). Similarly, swarming assays using the pooled mouse or individual human feces yielded single species of a dominant swarmer as identified using MALDI-TOF (Table 1, Supplementary Figure 1*B*). To test whether swarming bacteria are also present in preclinical models, we screened feces of mice exposed to DSS that caused acute colonic inflammation.[8,39] Swarmers (in feces) were uniformly absent in water-exposed mice, and present in DSS-exposed mice (Figure 1*E*). In a single experiment, we found "nearly identical isolates" (>99% identical, one contig of 5,107,194 bp [National Center for Biotechnology Information (NCBI) BioProject PRJNA558971]) from 2 different mouse fecal specimens – *Enterobacter* species SM1 from mice exposed to water and *Enterobacter* species SM3 from mice exposed to DSS (Supplementary Figure 2*A*). SM3 swarmed significantly faster compared with SM1. A quantitative PCR sequencing-based approach to accurately identify SM1 or SM3 like bacteria in feces showed a significant increase in its abundance during the evolution of DSS-induced colitis (>10,000 DNA copy number/μL) than the water-only group (Figure 1F). Taken together, using an agar-based assay, we were able to isolate nearly identical strains from a control and a mouse with colitis that exhibited striking differences in their swarming potential.

Swarming Enterobacter *species SM3 Abrogates Intestinal Inflammation in a Mouse Model of Colitis*

To determine the functional consequence of bacterial swarmers in the host, we administered the "near-identical" swarming competent SM1 or SM3 strains to mice with DSS-induced colitis. In comparison with SM1, SM3 is a hyperswarmer (Supplementary Figure 3*A*, Supplementary Video 1), but both strains have the same swim speed (Supplementary Figure 3*B–C*), surfactant production (Supplementary Figure 3*D*), and growth rate (Supplementary Figure 3*E*). In contrast to that observed with SM1, SM3 significantly protected mice from intestinal inflammation (Figure 2*A–F*). Comparison of clinical parameters showed that SM3 significantly protected from body weight loss (Figure 2*A*), increased colon length (Figure 2*B*), reduced the colonic inflammation score (Figure 2*D*), and had reduced expression of pro-inflammatory mediators compared with vehicle-treated colitic mice (Figure 2*E–F*). To test the mucosal healing capacity of swarming bacteria, we administered strains SM1 and SM3 to mice during the recovery phase of DSS exposure.[40] When compared with the vehicle, SM3 significantly improved weight gain and colon length with reduced total inflammation and fibrosis at the microscopic level (Supplementary Figure 4). In mice exposed to DSS, SM3, but not the swarming deficient mutants (SM3_18 and SM3_24), showed significant protection against weight loss (Figure 2*G*), colon length (Figure 2*H*), and inflammation (Figure 2*I*). SM3 and its isogenic transposon mutants (SM3_18 and SM3_24) only differed in swarming potential (Supplementary Figure 3*H*), but not swimming speed (Supplementary Figure 3*I–J*), surfactant production (Supplementary Figure 3*K*), or growth rate (Supplementary Figure 3*L*).

As a second model of colitis, we used a TLR5KO interleukin10R neutralization-induced colitis model of mice. SM3 also significantly protected from body weight loss (Supplementary Figure 5*A*), reduced spleen and colon weight (Supplementary Figure 5*B–C*), increased cecum weight (Supplementary Figure 5*D*), reduced serum keratinocyte-derived chemokine (KC) level and LCN2 level (Supplementary Figure 5*E–F*), reduced levels of fecal LCN2 (Supplementary Figure 5*G*), reduced myeloperoxidase activity (Supplementary Figure 5*H*), and had reduced the colonic inflammation score (Supplementary Figure 5*I*), when compared with the SM1.

A

		Disease*	
		+	-
Age	Mean (± SD)	51 ± 14	50 ± 11
	Median (Range)	52 (21-81)	51 (24-70)
Gender	Females	27	8
	Males	17	9

* Defined as individuals with clinically established *active* inflammatory bowel disease (Crohn's disease n = 14, or Ulcerative colitis n = 5), intestinal dysplasia (n =1), intestinal polyps (n = 23), intestinal reactive hyperplasia and inflammatory changes not otherwise specified (n = 1). All other diagnoses were considered "negative" for disease.

B

		Disease*	
		+	-
Swarming test †	Swarming	25	3
	Non-swarming	20	22

* One patient consented for the study specimen but did not undergo colonoscopy; One patient had a poor colonoscopy preparation and aspirate sample was not obtained; 9 fecal samples obtained from OpenBiome (Boston, MA), one of which was contaminated inadvertently with a laboratory swarming strain and was excluded from analysis.
† "Swarming" defined as swarming score ≥ 1; "Non-swarming" defined as swarming score = 0.

C

Statistic*	Value	95% CI†
Sensitivity	55.56%	40.00% to 70.36%
Specificity	88.00%	68.78% to 97.45%
Positive Likelihood Ratio	4.63	1.55 to 13.81
Negative Likelihood Ratio	0.51	0.35 to 0.72
Disease Prevalence	64.29%	51.93% to 75.39%
Positive Predictive Value	89.29%	73.63% to 96.13%
Negative Predictive Value	52.38%	54.88% to 77.91%

* Computed with: https://www.medcalc.org/calc/diagnostic_test.php
† CI, confidence interval

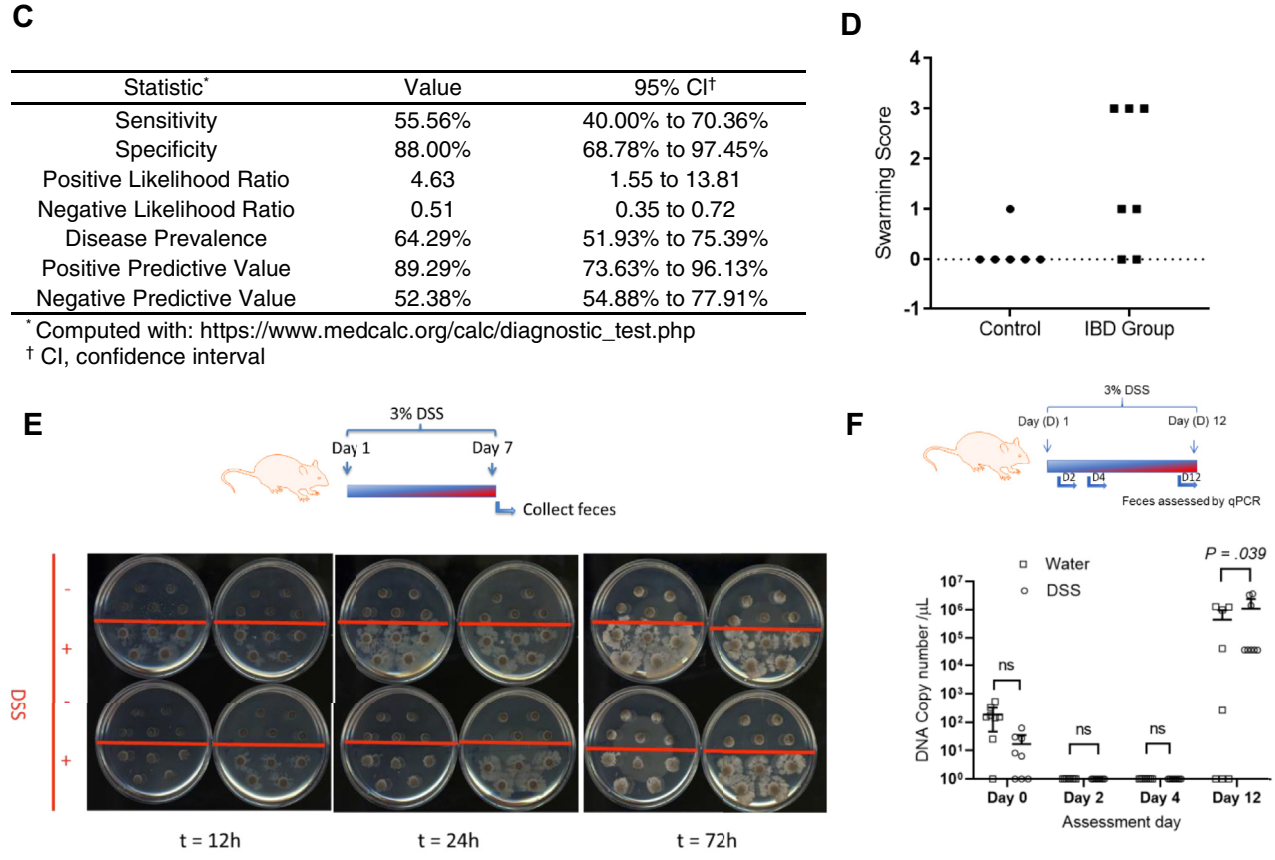


Figure 1. Effect of intestinal inflammation on bacterial swarming. (*A–C*) Human colonoscopy aspirates (n = 45 intestinal disease; n = 25 nondisease) were spotted on 0.5% agar plates and the swarming assay was performed. (*A*) Colonoscopic washes were obtained from individuals with active intestinal disease and matched controls. Swarming assays performed using aspirates were binned by disease as defined both clinically and using intestinal histopathology, where available. (*B*) Clinical demographics are described for the disease and nondisease population. (*C*) Swarming assays' clinical test characteristics. (*D*) Swarming assays (72 hours) of fecal samples collected from pigs with and without inflammatory bowel disease. Swarming scores: 0, no swarming; 1, swarming within 72 hours; 2, swarming within 48 hours; and 3, swarming within 24 hours or less (Control, n = 6; IBD, n = 7). (*E*) C57BL/6 mice (8 weeks old) were exposed to water or DSS water for 7 days (n = 4 per group). Fecal samples of control group (*above red line*) and DSS group (*below red line*) were collected for swarming assay. Swarming plates were scanned at 12, 24, and 48 hours. (*F*) C57BL/6 mice (8 weeks old) were exposed to water or DSS water for 12 days (n = 8 per group). Fecal samples were collected for DNA extraction and SM1/SM3-specific PCR analysis was performed, and DNA copy number ascertained. Data are represented as mean and 95% CI; significance was tested using Fisher exact test.

To determine if the bacterial abundance differed among mice exposed to SM3 or its mutants, we assessed the abundance of the strains in feces using qPCR. Using the DSS colitis model, on day 4, the levels of SM1, SM3, and its mutants present in feces were not significantly different (Supplementary Figure 6*A–C, F*). We chose to enumerate

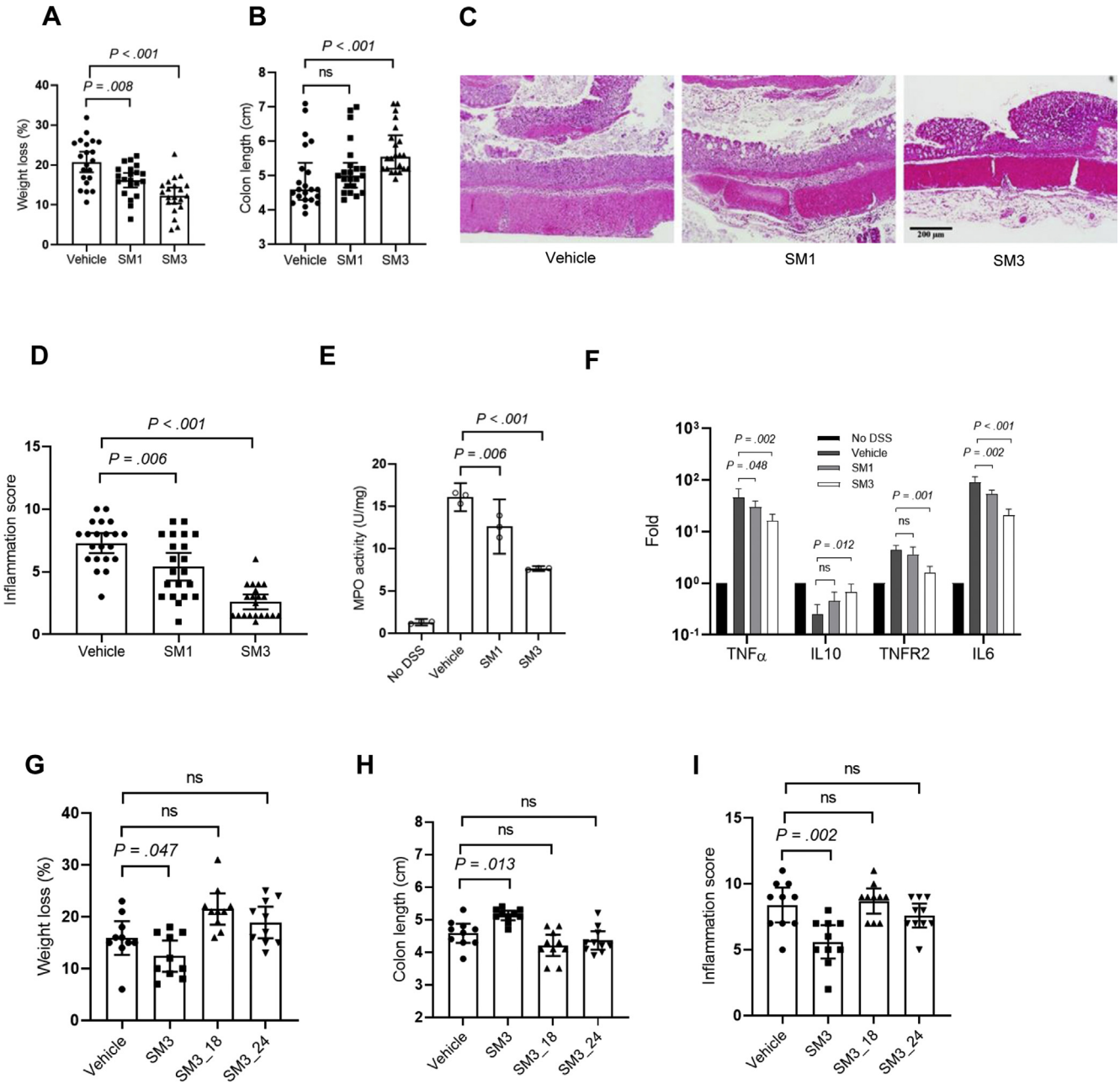


Figure 2. Effects of *Enterobacter* species SM strains on DSS-induced colitis in C57BL/6 mice. (*A–F*) 8-week-old mice were exposed to DSS water and treated with vehicle (LB), SM1, or SM3 by oral gavage for 10 days. (*A–B*) Weight loss (*A*) and colon length (*B*) (n = 21 per treatment group). (*C*) Representative images (100x magnification) of H&E–stained colonic section treated with vehicle (*left*), SM1 (*middle*), and SM3 (*right*). (D) Inflammation score (n = 21 per treatment group). (*E–F*) In a separate experiment, myeloperoxidase (MPO) enzyme activity was determined (n = 3, each in duplicate) (*E*). Colon total RNA (n = 4) was isolated and reverse transcribed to complementary DNA. Quantitative reverse transcription PCR (RT-qPCR) data show fold induction of messenger RNA (tumor necrosis factor [TNF]α, interleukin [IL]10, TNFR2, IL6). PCR was repeated in quadruplicate. The expression was normalized to internal control, TBP. The entire experiment was repeated n = 2 for reproducibility (*F*). (*G–I*) C57BL/6 mice (8 weeks old) were exposed to DSS water and administered vehicle (LB), SM3, or its mutants (SM3_18 or SM3_24) for 10 days. (*G–I*) Weight loss (*G*), colon length (*H*), and inflammation score (*I*) (n = 10 per treatment group). Unless otherwise noted, data are represented as mean and 95% CI, and significance was tested using 1-way analysis of variance (ANOVA) followed by Tukey post hoc test. (*B*) Data represented as median and interquartile range, and significance tested using Kruskal-Wallis followed by Dunn multiple comparisons test. TBP, TATA-Box Binding Protein.

bacterial levels in feces on Day 4 due to the equivalent pathologic conditions of mice, as defined by weight change, when treated with different strains. To identify if the loss of protection by SM3_18 could be related to slightly higher levels of its presence compared with SM3, although not significant, we performed a dose attenuation study, which demonstrated nonsignificant changes in either weight loss (Supplementary Figure 6*G*) or LCN2 levels (Supplementary

Figure 6*H*). In accordance with these results, a diverse set of commensal swarmers (*Bacillus subtilis* 3610 and *Serratia marcescens* Db10) and a clinical strain of *S marcescens* (isolated from the surface washing of a human dysplastic polyp) exhibited protection against DSS-induced inflammation in mice (Supplementary text and Supplementary Figures 7 and 8). Together, these data implicate or associate SM3 with swarming properties, as opposed to swarming-deficient strains, with anti-inflammatory activity.

SM3-Mediated Abrogation of Intestinal Stress is Microbiome Dependent

We used GF mice (GF/SPF) exposed to DSS and treated them with SM3 to determine if the anti-inflammatory role of SM3 depends on the conventional intestinal microbiome composition. This strain was unable to abrogate intestinal inflammation in GF/SPF mice (Figure 3*A*). We analyzed fecal samples of colitic mice (conventional and GF/SPF) with SM3 administered using 16S rRNA gene profiling. In contrast to GF/SPF mice, conventional mice feces showed specific enrichment of anaerobes belonging to the family S24-7 and Lactobacillaceae within SM3-treated mice when compared with vehicle mice (Figure 3*B*). Specifically, in conventional mice, we found a significant increase in the abundance of S24-7 with SM3 gavage compared with vehicle in DSS-exposed mice (Figure 3*C*). However, quantitative PCR analysis of the levels of S24-7 in the feces of DSS-induced colitis mice gavaged with SM1 or SM3_18 or SM3_24, which did not exhibit protection from intestinal inflammation, was significantly reduced (Figure 4*A*). In mice not exposed to DSS, the levels of S24-7 bacteria remain stable in the SM3-treated group compared with the untreated group (Figure 3*C*). We observed that enriched S24-7 negatively co-occurred within DSS-exposed conventional mice with pathogenic taxa such as the Peptostreptococcaceae and Enterobacteriaceae (Figure 3*D*). Together, these data suggest that protection from intestinal inflammation by SM3 is associated with the presence of a beneficial S24-7 group of bacteria.[41]

Enterobacter *species SM3 Promotes Growth of* M. intestinale *In Vitro*

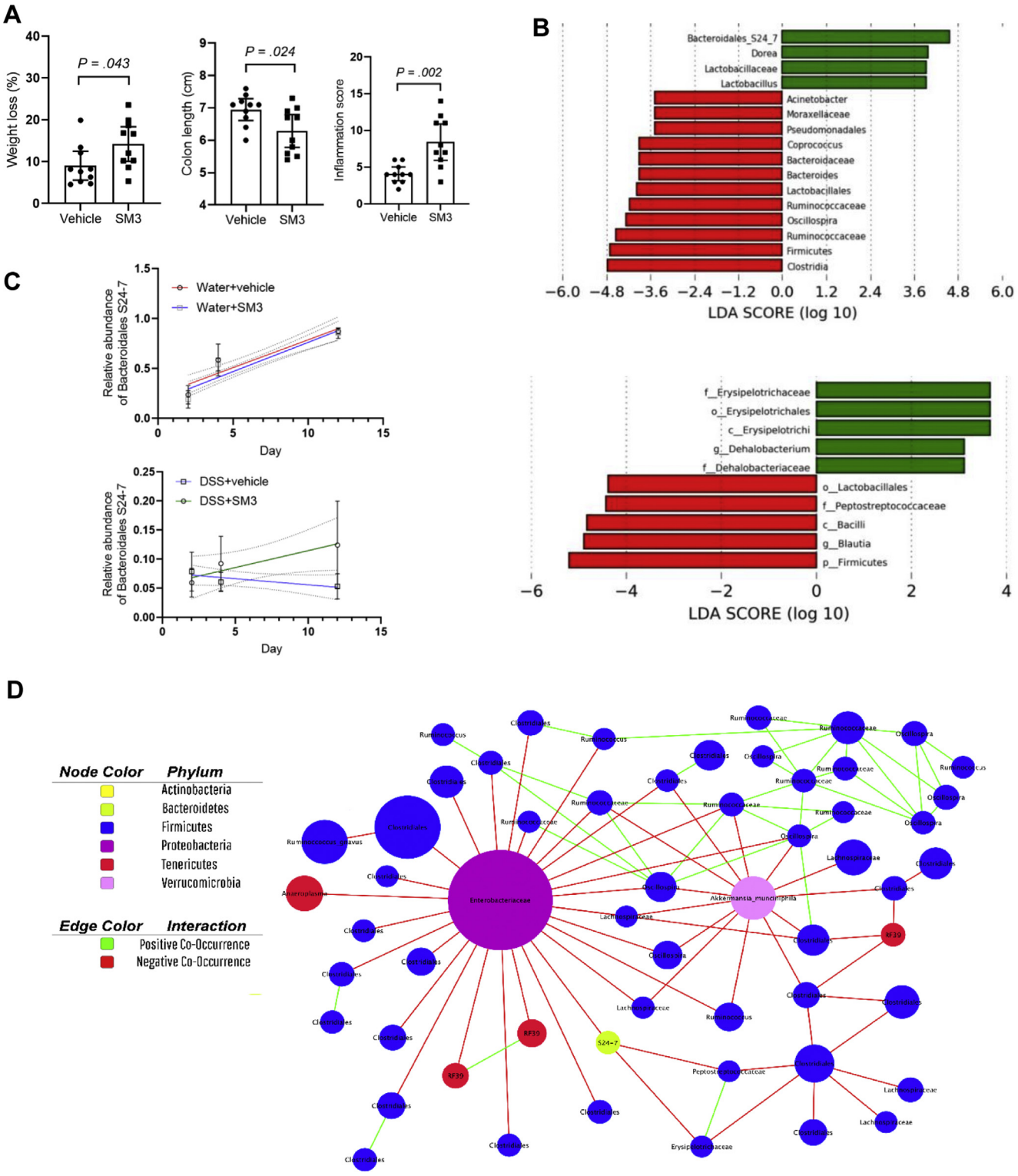
A recent study has reported the first cultured bacterium *M intestinale* (DSM 28989) belonging to the Bacteriodales S24-7 family.[42] We used this strain to delineate any potential interspecies interaction with SM3 using an in vitro coculture assay system. However, in precedence, we assessed if the strain *M intestinale* shared sequence homology to any of the S24-7 taxa identified in our fecal 16S rDNA profile. OTU_5, which we found in the highest abundance among all other OTUs representing S24-7 taxa, exhibited >96% identity to *M intestinale* (Supplementary Figure 9). Hence, we performed a broth-based coculture assay using this strain and SM3 or SM1 or SM3_18. Interestingly, the proportion of *M intestinale* during coculture was higher than its monoculture at any tested time point. SM3 and the partially swarming deficient strains, SM1 and SM3_18, had a 2- to 4-fold increase in DNA copy number/μL when analyzed using qPCR using S24-7–specific primers (Figure 4*B*).

We also designed and developed a plate-based coculture assay to compare the effects of swarming bacteria SM3 and swarming-deficient variants, SM1 or SM3_18, on the growth of *M intestinale*. In this assay, swarming plates harbored a central bore-well containing *M intestinale* that guarantees a direct or indirect interaction with the spreading bacteria on agar of the same plate. We sealed the plates so that the act of swarming generated an anaerobic environment suitable for the growth of *M intestinale*. At 64 hours, in congruence with the broth coculture assay results, we observed an increase in *M intestinale* counts with SM3, SM3_18, and SM1 (Figure 4*C*). To better understand the observed increase in *M intestinale* levels, we developed a separate plate-based coculture assay. In this assay, we physically separated the swarming region from the central bore-well containing *M intestinale* to prevent any direct or indirect interaction with the swarming bacterium. In this system, as the bacteria swarmed on the agar surface over 64 hours, oxygen levels were reduced. *M intestinale* showed no growth under the conditions tested (Figure 4*C*, Divided/Sealed). Overall, our results suggest that both planktonic and swarming cells of SM3, SM1, or SM3_18, when cocultured in vitro, can promote the growth of the S24-7 family (*M intestinale)*, independent of reduced oxygen concentrations in the environment. Coincidently, the development of significantly reduced oxygen concentrations in the environment is also observed in vivo but only with SM3 and not SM1 or the other SM3 mutant bacteria (Supplementary text and Supplementary Figure 10). Our results suggest that SM3 proximity to *M intestinale* is necessary to induce the growth of the latter species.

In this context, to understand if SM3 does swarm in vivo, we searched for the presence of transcriptomic markers in the feces that can be linked to SM3 swarming physiology. In agar-based studies of RNA sequencing of SM3 obtained from the edge of a swarming colony vs the preswarming colony at the center, a singular pathway was significantly upregulated – the lipid A biosynthetic pathway (fold change 3-fold, q value = 0.0376). Meta-transcriptomic analysis of feces from SM3-treated DSS-induced colitic mice identified a steady increase of the lauroyl acyltransferase transcript involved in Lipid A biosynthesis Day 4 and Day 12 when compared with Day 0 (Supplementary Figure 14*A*). However, heat-killed SM3 treatment showed a reduction in transcript abundance by Day 12. Other genes are known to be associated with swarming. The sigma factor FliA and nitrate reductase NarH were also enriched in SM3 vs heat-killed SM3 gavaged colitic mice (Supplementary Figure 14*B*, Supplementary text). Normal mice gavaged with SM3 or heat-killed SM3 did not show enrichment of these genes. Also, an ex vivo race assay on a colitic surface demonstrates the potential of bacterial swarming in vivo during colitis (Supplementary Figure 13, Supplementary text, and Supplementary Videos 2–5). Collectively, our data provides multiple lines of indirect evidence suggesting that bacterial swarming is a likely phenomenon in vivo, and a motility form that is necessary for the induction of *M intestinale* growth.

Discussion

Our study finds that intestinal inflammation itself promotes a protective niche that facilitates enrichment of bacterial swarmers. Despite the caveat that our approach might preclude the selection of swarmers that do not produce surfactant,[12] these pilot data indicate that collective spreading and swarming is a specific feature and potentially a biomarker of an intestinal pathology, as defined by harboring active intestinal inflammation or polyps. Surprisingly, however, these bacterial swarmers, when dosed in



sufficient abundance, abrogate intestinal inflammation in mice. Unfortunately, a limitation of our clinical study was that we did not analyze details of patient clinical history (eg, use of medications) to determine their relationship with the presence or quantitative performance of bacterial swarmers in feces.

We focused on a newly isolated bacterium, *Enterobacter* species SM3, which is resident to the intestinal microflora of mice. In vivo, SM3, but not SM1, or SM3 swarming-deficient mutants (poor swarmers), influenced the specific enrichment of the S24-7 group of bacteria. Notably, the family of S24-7 (*Muribaculaceae*) is known to repair barrier function in inflamed mice intestines.[41,43] However, the in vitro coculture experiment proved that a close interaction between SM3 and the S24-7 group of bacteria is essential for its enrichment. Thus, we hypothesized that it is the relative hyperswarming activity of SM3 (but not the weak swarming SM1 or SM3 mutants) that may facilitate close interaction with the S24-7 group of bacteria in vivo. Further support of this hypothesis comes from the bacteria's ability to swarm on a mucosal surface afflicted by colitis but not on the normal mucosal surface (ex vivo mucosal race assay), and from the meta-transcriptomic mining of swarming-associated genes in colitic mice administered SM3 (but not its heat-killed counterpart). The present mechanism implicates swarming SM3 in enhancing S24-7 (*Muribaculaceae*), which then suppresses host inflammation. Nevertheless, we do not exclude other direct or indirect effects of the swarming SM3 on mucosal inflammation and healing. However, if present, it would assist in suppressing host inflammation in conjunction with enrichment of the S24-7 group of bacteria in the gut.

Swarming bacteria secrete surfactants, such as surfactin, that facilitate motility on a solid surface.[12] Surfactin is known to attenuate 2,4,6-Trinitrobenzenesulfonic acid (TNBS)-induced colitis, possibly by differentially regulating anti-inflammatory and pro-inflammatory cytokines.[44] However, none of the isogenic pairs showed significant difference in surfactant production, suggesting that the observed protection was not due to secreted surfactin (Supplementary text). Transpositions in SM3_18 and SM3_24 were found to be located within the putative structural genes encoding N6-hydroxylysine O-acetyltransferase or aerobactin synthesis protein (*iuc*B) and isocitrate/isopropylmalate dehydrogenase/adenosine diphosphate-ribose pyrophosphate of COG1058 family, respectively (Supplementary text). Nevertheless, transposon integration in SM3_18 led to a polar insertion that will only disrupt the expression of downstream genes *iucC*, *iucD*, and *iutA* located within the operon, hampering aerobactin synthesis only. Fundamentally, genes *iucD* and *iutA* aid in iron acquisition in bacteria during nutrient-limiting condition.[45] A single study has also shown the dependence of bacterial phytopathogen *Pantoea stewartii* swarming on aerobactin synthesis.[46] However, in this context, we did not observe any changes in fecal iron levels in colitic mice exposed to either WT SM3 or SM3_18 (Supplementary text), negating the iron effects in the suppression of inflammation.

In a GF/SPF condition, the loss of protection by SM3 allows us to speculate on the role of a full-spectrum intestinal microbiome in the observed effect. Oral gavage of SM3 in conventional colitic mice enriched beneficial anaerobes. Because intestinal inflammation creates a shift from anoxic to oxic[47] (Supplementary Figure 10*A*), it was unexpected to find enrichment of obligate anaerobes such as Bacteriodales S24-7 in SM3-treated mice. We observed SM3-fed colitic mice had significantly lower oxygen concentration than the colitic mice treated with swarming-deficient variants. We conjectured that the possible role of swarming movement of SM3, if occurring in vivo, could be reducing oxygen concentration as also observed in vitro. It was further corroborated by the increase in anaerobic taxa in the feces of GF/SPF mice treated with SM3 (Supplementary Figure 11).

Nevertheless, a steady increase of S24-7–specific OTUs in SM3-treated DSS-colitic mice pointed toward a potential mechanism underlying the observed protection. Hence, we

Figure 3. Effects of SM3 on the intestinal microbiota of GF/SPF and conventional mice. (*A*) C57BL/6 GF/SPF mice (5 weeks old) were exposed to DSS water and treated with vehicle (LB) or SM3 for 6 days. (*A*) Weight loss (*left*), colon length (*middle*), and inflammation score (*right*) (n = 10 per treatment group). (*B*) Linear discriminant analysis Effect Size (LEfSe) plot of taxonomic biomarkers identified using feces of SM3-treated conventional (n = 10) (*upper*) and GF/SPF (n = 10) (*lower*) colitic mice on day 12 and day 6, respectively, as compared with vehicle (n = 10). *Green bars* and *red bars* indicate bacterial enrichment within the SM3-treated and vehicle groups, respectively. All taxa that yielded a linear discriminant analysis (LDA) score >3.0 are presented. (*C*) Relative abundance of S24-7 in the feces from DSS (*lower*) and control (*upper*) mice treated with SM3 or vehicle (n = 8 per treatment group). Linear regression line was fit to show the trend of the change (*dotted lines*, 95% confidence bands). The slope of the SM3-treated group is similar to the vehicle in water control group ($P = .783$), but significantly different in the DSS group ($P = .018$). (*D*) Co-occurrence network plot showing strong positive and negative correlations between OTU abundances. All networks were generated with CoNet and visualized in Cytoscape. Processing was applied to the dataset with CoNet. Input filtering constrained the minimum occurrence of OTUs and considered only those present in at least 50% of samples. Standardization normalized dataset columns. Networks were constructed using Spearman correlation methods with threshold setting at 0.9, Bray Curtis dissimilarity at the automatic threshold setting, and Kullback-Leibler dissimilarity at the automatic threshold setting; the edge selection parameter was set to 30 for the strongest positive and negative correlations. Randomization steps included permutations and bootstraps with filtering of unstable edges and Benjamini-Hochberg procedure with a *P* value of .05. Node clusters with less than or equal to 3 edges were not shown in the final network. Edge coloration indicates copresence in *green* or mutual exclusion in *red*. Nodes were colored by taxonomic phylum and labeled by the highest taxonomic ranking available. Unless otherwise noted, data are represented as mean and 95% CI, and significance tested using a 2-tailed Student *t* test.

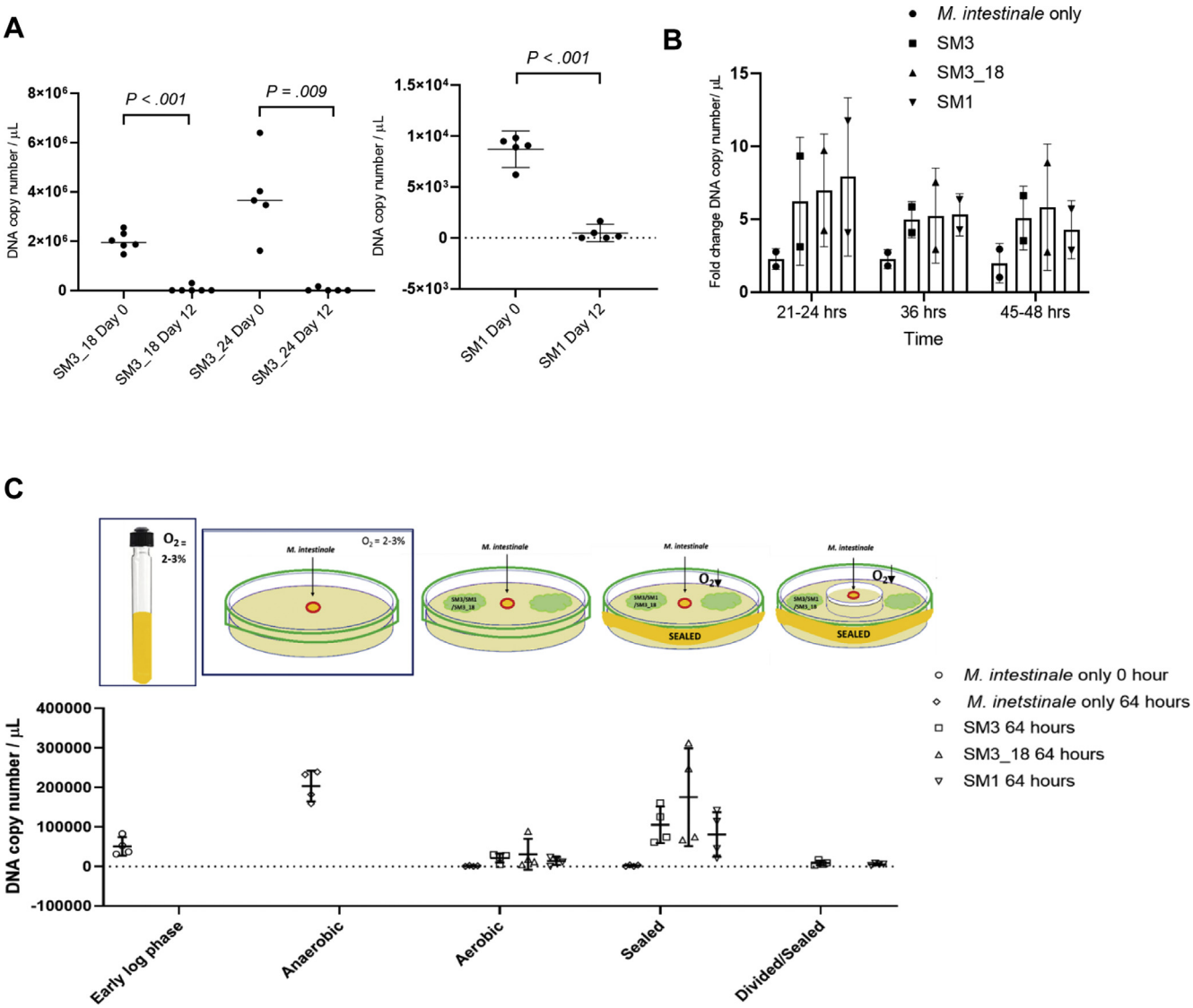


Figure 4. Effect on S24-7 levels in the presence of SM3 and the insufficient (or inefficient) swarming variants in vivo and in vitro. (*A*) 8-week-old mice (n $\geq$ 5 per treatment group) were exposed to DSS water and treated with SM3_18, SM3_24, and SM1 by oral gavage for 12 days. Total DNA was extracted from feces collected on day 0 and day 12, processed and assessed using qPCR. Five nanograms of total DNA in conjunction with S24-7 specific primers were used to quantify bacterial copy numbers. In each assay, DNA copy number/μL was calculated based on an internal standard curve. (*B-C*). In vitro coculture assays using *M intestinale* cells grown in chopped meat medium under anaerobic condition until early log phase (OD_{600} $\approx$ 0.5) were used. (*B*) Fold change DNA copy number/μL relative to *M intestinale* monoculture. In broth-based assay, 2 μL of early log phase cells of SM3, SM3_18, or SM1 were added to *M intestinale* cells and mixed cells or monoculture of *M intestinale* was collected at regular intervals (21–24, 36, 45–48 hours). (*C*) In a swarming plate–based assay, early log phase *M intestinale* was transferred in the bore-well and SM3, SM3_18, or SM1 was allowed to swarm either under aerobic or sealed condition at 37ºC and relative humidity $\approx$ 50%. Plates were sealed using parafilm to create and maintain anaerobiosis due to the act of swarming. *M intestinale* grown under anaerobic condition was used as a positive control. In divided/sealed condition, swarming region was physically separated from the bore-well containing *M intestinale* and sealed using parafilm. Closed boxes represent incubation in an anaerobic chamber. DNA extracted from equal volume of culture and resuspended in equal volume of Tris-EDTA (TE) buffer was used for qPCR in conjunction with *M intestinale*–specific primers. (*A*) Data are represented as mean and 95% CI, and significance tested used paired *t* test. (*B–C*) Data represented as mean (±SD) (n = 2 independent experiments and 2 technical replicates for each).

designed a broth and plate-based coculture assay to identify possible interactions between SM3 and the first cultured bacterium belonging to the S24-7 family, *M intestinale*. Both SM3 and the less swarming variants promoted the growth of *M intestinale* in coculture assay. However, linking this observation with a decrease in the levels of S24-7 in the fecal DNA obtained from SM1, SM3_18, and SM3_24 led us to speculate about the essential role of swarming by SM3 in exhibiting protection. We conjectured that in addition to an anaerobic

Table 1. Bacterial Strains Isolated and Used in This Study

Bacterial strains identified from luminal contents and isolated on swarming agar[a]		
Strain isolated	Swarming	Source
Escherichia coli[f]	+	Human IBD
Escherichia coli[f]	+	Human IBD
Escherichia coli	+	Human anal fistula
Klebsiella pneumoniae	+[b]	Human IBD
Klebsiella pneumoniae	-[c]	Healthy human
Citrobacter koseri	+	Human IBD
Morganella morganii	-[d]	Human IBD
Serratia marcescens	+	Human adenomatous polyp
Proteus mirabilis	+[e]	Mouse colitis
Proteus mirabilis	+[e]	Mouse colitis
Enterobacter species[f]	+	Mouse (DSS colitis)
Enterobacter species[f]	+	Mouse (TNBS colitis)

Bacterial strains used in this study		
Organism	Description	Reference
Enterobacter species SM1	A clinical isolate from feces of normal mice	This study
Δ*motA* SM1	A flagella motor function abrogated mutant of SM1, motA::kan	This study
Δ*flhE* SM1	A flagella-associated gene involved in swarming, flhE::FRT:Kan:FRT	This study
HS2B SM1	A hyperswarming variant of SM1 generated by serial passage on swarming agar	This study
Enterobacter species SM3	A clinical isolate from feces of DSS-colitis mice	This study
SM3_18	A transposon mutant of SM3, putative aerobactin synthesis gene iucB::Tn::kan	This study
SM3_24	A transposon mutant of SM3, putative isocitrate/isopropylmalate dehydrogenase/ADP-ribose pyrophosphate gene::Tn::kan	This study
Serratia marcescens	A clinical isolate from human adenomatous polyp.	This study
Bacillus subtilis		
3610	A WT isolate	Kearns & Losick, 2003[9,g]
DS215	A swarming defective mutant of 3610, swrA::tet	Kearns et al[10,g]
Serratia marcescens		
Db10	A WT isolate	Pradel et al[11,g]
JESM267	A serrawettin W2 defective mutant of Db10, swrA::miniTn5-Sm	Pradel et al[11,g]
Salmonella enterica serovar Typhimurium		
ATCC 14028	A WT isolate	
Δ*fliL*	A swarming deficient mutant of *S enterica*, fliL::FRT	This study
Muribaculum intestinale YL27	A strict anaerobe isolated from cecal content of mice	Lagkouvardos et al[42]

ADP, adenosine diphosphate; TNBS, 2,4,6-Trinitrobenzenesulfonic acid.
[a]Human or mouse feces was subject to the swarming assay and any swarm colony detected within 24 h was swabbed for strain identification. In addition, delayed swarmers were classified as negative but their swarm edge also yielded single species.
[b]Feces from patient with clinically controlled Crohn's disease with moderate surfactant edge detected at 74 h.
[c]Classified as nonswarmer, however, a very minimal surfactant edge present at 24 h and no progression thereafter.
[d]Feces from patient with clinically controlled Crohn's disease with surfactant edge detected at 48 h.
[e]Mouse model: Msh2/-loxPTgfbr2 loxp Villin-cre.
[f]Also confirmed using Illumina Sequencing (PacBio).
[g]References cited in the Supplementary Materials.

environment generated by the act of swarming on the agar plate, all the tested strains either required a direct cell-cell contact or produced a secretome, which promoted the growth of *M intestinale*. This was further validated by the negative outcome by design of a plate-based assay that allowed physical separation of swarming SM3 from *M intestinale* and created an anaerobic condition in the system suitable for the growth of *M intestinale*.

Based on the evidence of swarming on a mucosal surface, we conclude that swarming of SM3 in vivo may facilitate close spatial interaction with the S24-7 group of bacteria. SM3 may aid in re-establishing hypoxia and, consequently, creating an optimal condition for the enrichment of S24-7 and other anaerobes in a specific microenvironment. In summary, our work demonstrates the unique and unprecedented role that bacterial swarmers play in intestinal homeostasis. We find the potential for a new personalized "probiotic" approach stemming from the ability to isolate and bank swarming microbes during colitic episodes.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2021.03.017.

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Conflicts of interest
Sridhar Mani, Libusha Kelly, and Hao Li filed a U.S. patent application (Application No. 15/765,513). The remaining authors disclose no conflicts.

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