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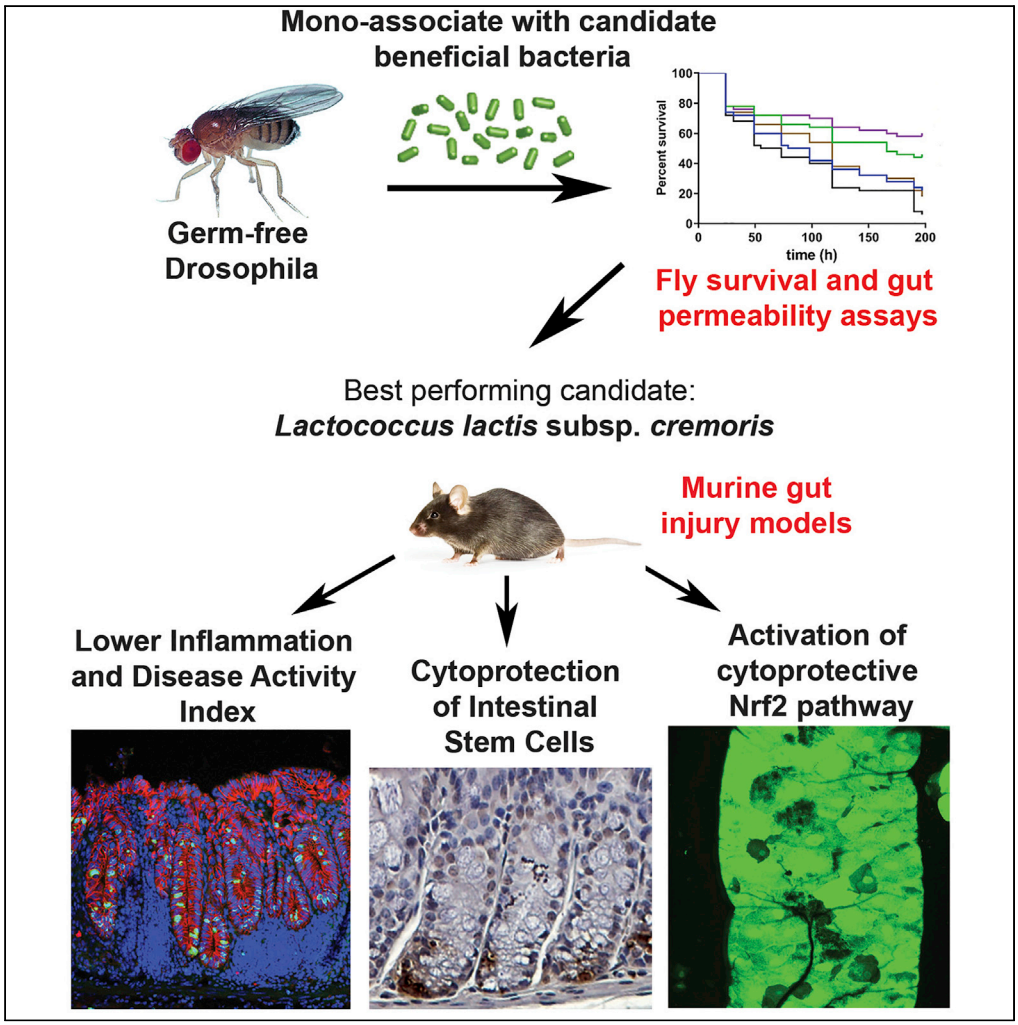

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HIGHLIGHTS

Drosophila can be used as an animal model to screen for beneficial bacteria

Lactococcus lactis subsp. *cremoris* elicited potent cytoprotection in the fly gut

L. lactis cremoris elicited anti-inflammatory activity in a mouse colitis model

L. lactis cremoris activated the cytoprotective Nrf2 pathway in flies and mice

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Lactococcus Lactis Subsp. *cremoris* Is an Efficacious Beneficial Bacterium that Limits Tissue Injury in the Intestine

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SUMMARY

The use of beneficial bacteria to promote health is widely practiced. However, experimental evidence corroborating the efficacy of bacteria promoted with such claims remains limited. We address this gap by identifying a beneficial bacterium that protects against tissue damage and injury-induced inflammation in the gut. We first employed the *Drosophila* animal model to screen for the capacity of candidate beneficial bacteria to protect the fly gut against injury. From this screen, we identified *Lactococcus lactis* subsp. *cremoris* as a bacterium that elicited potent cytoprotective activity. Then, in a murine model, we demonstrated that the same strain confers powerful cytoprotective influences against radiological damage, as well as anti-inflammatory activity in a gut colitis model. In summary, we demonstrate the positive salutary effects of a beneficial bacterium, namely, *L. lactis* subsp. *cremoris* on intestinal tissue and propose the use of this strain as a therapeutic to promote intestinal health.

INTRODUCTION

Beneficial microbes are viable microorganisms that induce an advantage to health when ingested in sufficient amounts (Salminen et al., 1999). These bacteria can prompt a change in the diversity and metabolic activity of the gut microbiota (Bron et al., 2012), can modulate innate and adaptive immune responses (Scholz-Ahrens et al., 2007; Yan and Polk, 2011), or can significantly improve epithelial barrier function (Anderson et al., 2010; Seth et al., 2008). Despite the reported beneficial influence of some microbes, there is still a scarceness of empirical data to substantiate the claims made my marketed microbial products. This poses a considerable challenge to consumers when selecting a suitable microbe as a potential therapeutic intervention to treat a given ailment. Thus there is an urgent need to expand the primary scientific literature that experimentally substantiates the proposed uses of beneficial microbes.

There is also a pressing need to develop efficient and faithful discovery platforms for identifying strains of beneficial microbes. The use of cultured mammalian intestinal epithelial cells for this purpose has many evident caveats, including the lack of complex tissue physiology, absence of specialized cell types, as well as the nonappearance of a mucous layer as a barrier to prevent direct contact of cells and microbes. On the other hand, screening for candidate beneficial microbes *in vivo* using the mouse model is prohibitively expensive with vast numbers of mice likely required for discovery and generation of statistically significant data. To address these challenges, our research group and others recently reported on conserved molecular mechanisms of host cell and microbe interactions in the intestines of both mice and *Drosophila* (Jones et al., 2013; Neish and Jones, 2014). Indeed, the conserved nature of host cell and microbe interactions between mice and *Drosophila* has been widely reported, with examples including, but not limited to, juvenile growth during undernutrition (Schwarzer et al., 2016; Storelli et al., 2011), restitutive signaling pathways that respond to bacterial infection (Buchon et al., 2009, 2010; Chakrabarti et al., 2012; Zhou et al., 2015), microbiota-induced host gene expression (Broderick et al., 2014; Dantoft et al., 2013), the role of NADPH oxidases in epithelial response to mucosal dysbiosis (Grasberger et al., 2015; Ha et al., 2009), and normal tissue development and stem cell response to gut microbes, which was recently comprehensively reviewed by Bonfini et al. (Bonfini et al., 2016). In our studies, we showed that lactobacilli elicit cytoprotection in the intestines of mice and flies by a similar mechanism involving the activation of the cytoprotective Nrf2 pathway (Jones et al., 2015; Sinha et al., 2017). Owing to the transkingdom conservation of the functional elements that mediate the influences of beneficial

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microbes on host cells, we exploited and developed the use of the *Drosophila* model as an accessible and genetically tractable *in vivo* discovery platform for discovering beneficial bacteria.

The majority of promoted beneficial bacteria are lactic acid bacteria (LAB), with species from the *Lactobacillus* genus especially prevalent in marketed capsular and synbiotic preparations. Of the *Lactobacillus* species, *Lactobacillus casei* and *Lactobacillus rhamnosus* are arguably the most widely marketed beneficial bacteria, with investigative efforts focused on identifying the functional elements within specific strains of these species that mediate in their beneficial influences (Jones, 2016). Indeed, our research group has been at the forefront of generating a substantial body of data describing the molecular mechanisms whereby *L. rhamnosus* GG elicits its beneficial influences on the host (Alam et al., 2014, 2016; Ardita et al., 2014; Jones et al., 2012; Wentworth et al., 2010, 2011). By comparison, few (if any) species from the *Lactococcus* genus have been experimentally demonstrated to exhibit beneficial influences on the host. Rather, lactococci are used in the dairy industry in the manufacture of fermented dairy products with *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* commonly used as starter cultures in the production of cheese (Song et al., 2017). Importantly, because of its historical use in food fermentation, *L. lactis* has been provided the generally recognized as safe (GRAS) status by the US Food and Drug Administration (Wessels et al., 2004).

Herein, we describe the discovery of a strain of beneficial bacteria, namely, *L. lactis* subsp. *cremoris* ATCC 19257 (*L. lactis* subsp. *cremoris*) that we show confers potent cytoprotective and anti-inflammatory influences in the mouse intestine. We first discovered the cytoprotective properties of *L. lactis* subsp. *cremoris* in an *in vivo* screen using the *Drosophila* model that assess the potential beneficial influences of candidate beneficial bacteria. In assays measuring cytoprotection and gut permeability in the fly, *L. lactis* subsp. *cremoris* exhibited significantly stronger beneficial influences than any other tested bacteria. In the murine model, *L. lactis* subsp. *cremoris* protected mice from irradiation-induced intestinal tissue damage and lessened inhibited disease parameters induced by dextran sodium sulfate (DSS)-induced colitis. Importantly, *L. lactis* subsp. *cremoris* exhibited significantly stronger beneficial influences compared with *L. rhamnosus* GG, which is the most extensively studied beneficial microbe. Together, our data identify a beneficial bacterium, namely, *L. lactis* subsp. *cremoris* ATCC 19257 that exhibits potent anti-inflammatory and cytoprotective effects on the fly and mammalian intestine. We propose that the empirical data generated and reported in this article provide experimental basis for the use of *L. lactis* subsp. *cremoris* as a beneficial bacterium in the clinical environment.

RESULTS

Lactococcus lactis subsp. *cremoris* ATCC 19257 Confers Potent Cytoprotective Effects on the *Drosophila* Intestine in Response to Oxidative Challenge

We previously showed that a strain of *Lactobacillus plantarum* that we isolated from the midgut of *Drosophila* has cytoprotective influences against paraquat-induced oxidative challenge (Jones et al., 2015). This was in contrast to feeding other commensal bacteria isolated from the fly intestine that did not exhibit cytoprotection in the same assay. We thus rationalized that *Drosophila* mortality following exposure to paraquat may be a faithful assay to identify bacteria that elicit beneficial influences on the host organism. To establish this notion, we assessed the survival in response to paraquat of 5-day-old germ-free adult *Drosophila* (Luo et al., 2016) gnotobiotically mono-colonized with candidate LAB (Table 1). Of those tested, flies gnotobiotically mono-colonized with pure cultures of *L. lactis* subsp. *cremoris* ATCC 19257 were highly protected against oxidative injury as determined by the increased survival and significantly fewer apoptotic cells in the posterior midgut in response to paraquat (Figures 1A–1D). In addition, to determine the extent to which bacteria that have already been shown to elicit cytoprotection in mammalian models could also protect against paraquat toxicity assay in *Drosophila*, thereby showing that the fly assay is suitable for identifying probiotic bacteria that would potentially be effective in mammals, we gnotobiotically colonized *Drosophila* with the well-studied probiotic *L. rhamnosus* GG. We found that *L. rhamnosus* GG also elicited cytoprotection against paraquat toxicity in the fly model, albeit to a milder extent compared with *L. lactis* subsp. *cremoris* (Figures 1A–1D). Importantly, other LAB strains tested, including *Lactobacillus gasseri* ATCC 19992 and *Lactobacillus acidophilus* ATCC 4356, and those listed in Table 1, did not elicit cytoprotection in this assay, showing that the capacity of bacteria to elicit beneficial influences on the host is specific and variable within clades of bacterial taxa. We also assessed the capacity of the candidate bacterial strains to modulate gut permeability in adult *Drosophila*. This is done by an assay developed by Rera et al. (2012) that detects the amount of a colored dye (inert blue food dye) that

Bacterial Strain	American Type Culture Collection (ATCC)
Lactobacillus acidophilus	ATCC 314
L. acidophilus	ATCC 4356
Lactobacillus fermentum	ATCC 9338
Lactobacillus gasseri	ATCC 19992
Lactobacillus leichmannii	ATCC 7830
Lactobacillus paracasei subsp. *paracasei*	BAA-52
Lactobacillus rhamnosus GG	ATCC 53103
L. rhamnosus	ATCC 7469
L. rhamnosus	ATCC 9595
L. rhamnosus	ATCC 53103
Lactococcus lactis	ATCC 11454
L. lactis	ATCC 19435
L. lactis	ATCC 49032
L. lactis subsp. *cremoris*	ATCC 19257

Table 1. List of Candidate Beneficial Bacteria Used to Mono-Associate Germ-free *Drosophila* and Screened for Their Capacity to Protect *Drosophila* against Paraquat-Induced Death or Paraquat-Induced Intestinal Damage in a "Smurf" Assay.

Some of the listed bacteria were also used in assays to test for cytoprotection in murine gut injury assays.

translocates from the gut lumen to the open hemolymph of flies, which is the fluid that occupies the fly hemocoel and which is equivalent to blood in invertebrates. Specifically, 5-day-old germ-free male *w1118* flies were mono-colonized with pure cultures of candidate bacteria for 3 days. These flies were then exposed to paraquat diluted in blue food dye solution. Damage to intestinal tissue induced by paraquat results in loss of gut barrier integrity and translocation of the blue dye to the hemocoel, which is salient and visible under a light dissecting microscope (Figure 1E). Corroborating survival assays in Figure 1A, mono-association with *L. lactis* subsp. *cremoris* resulted in significantly fewer flies with blue dye translocation to the hemolymph than germ-free flies (Figure 1F). In addition, mono-association with *L. rhamnosus* GG resulted in significantly fewer flies with blue dye, albeit to a lesser extent compared with *L. lactis* subsp. *cremoris*, whereas flies mono-associated with non-cytoprotective bacteria could not significantly inhibit blue dye translocation to the hemolymph in the following paraquat exposure (Figure 1F). Together, these data demonstrate the first evidence for the beneficial properties of *L. lactis* subsp. *cremoris* ATCC 19257, beneficial properties that were markedly more efficacious in the *Drosophila* compared with *L. rhamnosus* GG or other LAB tested.

L. lactis subsp. *cremoris* ATCC 19257 induces the activation of the Nrf2 signaling pathway in the *Drosophila* intestine by a mechanism that does not involve the detectable generation of reactive oxygen species (ROS).

We previously demonstrated that *L. plantarum* contact with cells induces ROS generation in *Drosophila* enterocytes (Jones et al., 2013), and that cellular ROS enzymatically generated in response to contact with *L. plantarum* has salutary effects against exogenous insults to the intestinal epithelium via the activation of Nrf2-responsive cytoprotective genes (Jones et al., 2015). These observations were recently corroborated by Lemaitre's group (Iatsenko et al., 2018). To determine whether *L. lactis* subsp. *cremoris* also induces the generation of ROS in the *Drosophila* midgut cells, thereby postulating a mechanism whereby *L. lactis* subsp. *cremoris* elicits cytoprotection, we used a class of ROS-sensitive hydrocyanine dyes (hereafter referred to as hydro-Cy3) to detect lactobacilli-induced ROS generation in larval midgut cells (Kundu et al., 2009). Intriguingly, whereas *L. plantarum* and *L. rhamnosus* GG induced high levels of ROS in larval midgut epithelial cells, *L. lactis* subsp. *cremoris* did not induce any detectable amounts of ROS generation in the same cells (Figure 2A). However, we also measured the capability of *L. lactis* subsp. *cremoris* to

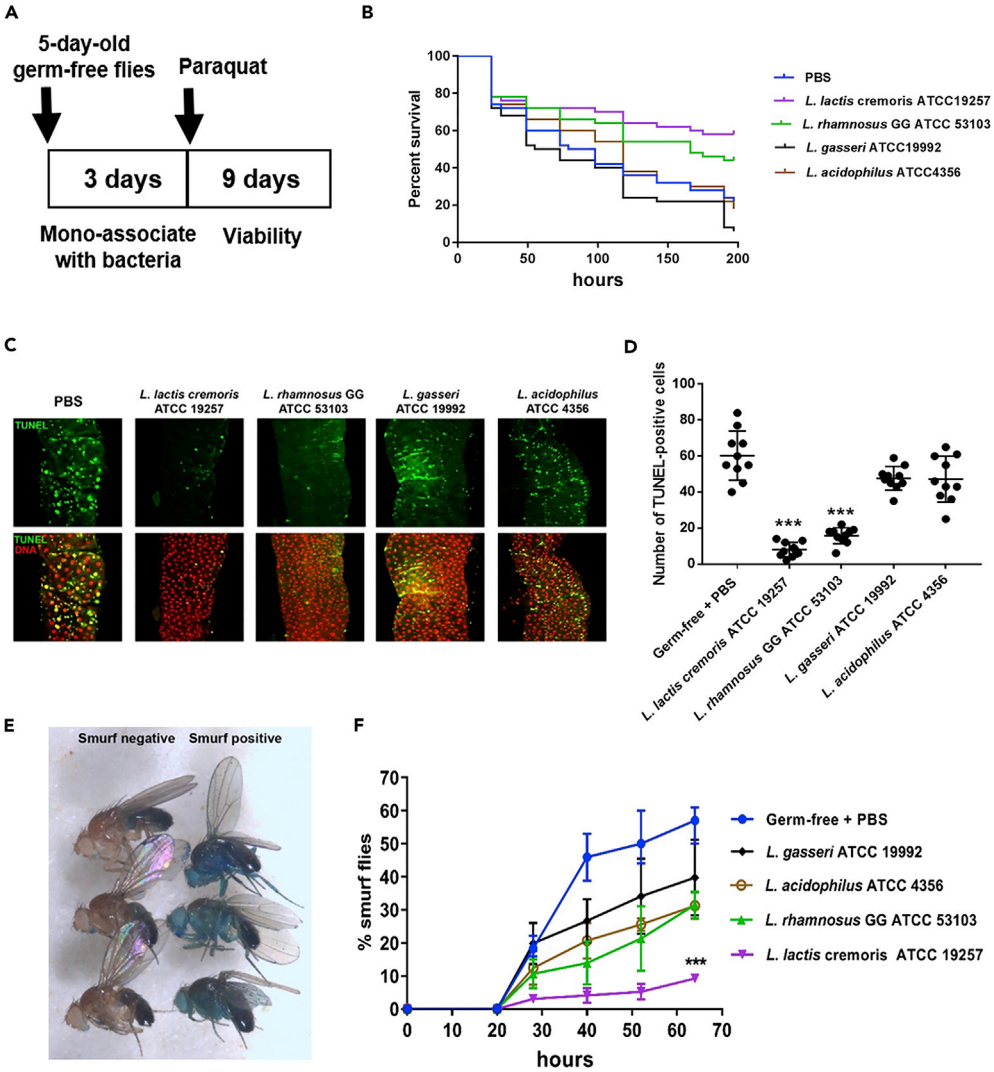

Figure 1. *Lactococcus lactis* Subsp. *Cremoris* ATCC 19257 Confers Potent Cytoprotective Effects in the *Drosophila* Intestine in Response to Oxidative Challenge

(A) Graphical representation of experiments conducted to generate data presented in (B–D).

(B) Relative survival of germ-free adult *Drosophila* gnotobiotically mono-colonized with the indicated lactic acid bacteria in response to paraquat challenge. Note only significantly enhanced survival of flies mono-associated with *L. lactis* subsp. *cremoris* ATCC 19257 (log rank test for germ-free (g-f) versus g-f + *L. lactis* subsp. *cremoris*, $p \leq 0.0006$, n = 50), or mono-colonized with *Lactobacillus rhamnosus* GG (log rank test for g-f versus g-f + *L. rhamnosus* GG $p \leq 0.0202$, n = 50).

(C) TUNEL analysis of posterior midgut tissues dissected from *Drosophila* described in (A) at 24 h following paraquat challenge. Note the presence of TUNEL-positive cells in the Posterior 3 (P3) section of the midgut in response to paraquat.

(D) Numeration of TUNEL-positive cells per midgut examined in (C). Data are represented as mean ± SEM. Nonparametric unpaired t test ***$p < 0.001$, n = 10.

(E) Example of adult *Drosophila* used to assess gut permeability via a Smurf assay. Germ-free adult *Drosophila* flies were gnotobiotically mono-colonized with the indicated lactic acid bacteria for 3 days. Thereafter, flies were subjected to a paraquat solution (spiked with inert blue food dye), which induces a leaky gut phenotype within 24–48 h. Flies that have a leaky gut are identified by exhibiting a visible blue color throughout their hemocoel within the body cavity.

(F) Numeration of germ-free flies with visible blue color throughout their hemocoel as described in (D), in response to feeding of the indicated bacteria for 5 days, followed by being subjected to 17.5% paraquat solution and inert blue dye for up to 72 h. Note significantly fewer flies with visible blue color throughout their hemocoel following pretreatment with *L. lactis* subsp. *cremoris* ATCC 19257. Data are represented as mean ± SEM. Nonparametric unpaired t test ***$p < 0.001$, n = 50.

induce the activation of the cytoprotective Nrf2 pathway in flies using a GFP reporter fly bearing an antioxidant response element-dependent promoter (*gstD1*-GFP) that responds to Nrf2 nuclear translocation (known as CncC in *Drosophila*) (Sykiotis and Bohmann, 2008). We found that *L. lactis* subsp. *cremoris* is a potent activator of the CncC pathway to at least an equal extent as *L. plantarum* and *L. rhamnosus* GG (Figure 2A). Furthermore, we detected CncC-pathway-responsive gene transcript enrichment of *gstD1* in the midgut of third instar larvae mono-associated with *L. lactis* subsp. *cremoris* to levels that were significantly higher than those detected for *L. plantarum* and *L. rhamnosus* GG (Figure 2B). To corroborate these observations in mammalian cells, we tested the capacity of *L. lactis* subsp. *cremoris* to generate ROS in cultured enterocytes. Cells were loaded with the hydro-Cy3 dye and then overlaid with LAB. *L. rhamnosus* GG induced potent activation of ROS generation consistent with previous reports (Jones et al., 2013), whereas there was no detectable ROS generation in cells overlaid with *L. lactis* subsp. *cremoris* (Figure 2C). However, similar to *L. rhamnosus* GG (Jones et al., 2015), we detected transcript enrichment of Nrf2-responsive genes in the colon of germ-free mice colonized with *L. lactis* subsp. *cremoris* (Figure 2D). These data show that *L. lactis* subsp. *cremoris* may elicit beneficial influences on the host intestine by a different mechanism from that shown to function in the beneficial influences of *L. plantarum* and *L. rhamnosus* GG (Jones et al., 2015).

Ingestion of *Lactococcus lactis* subsp. *cremoris* Is Cytoprotective against Radiological Insult in Mice

Identification of beneficial bacteria that dampen the symptoms of chronic inflammatory diseases of the intestinal tract is of intense investigative focus. We thus examined the extent to which *L. lactis* subsp. *cremoris* elicited cytoprotection in the intestine against radiological insult. Mice were fed either vehicle control, or 1×10^8 colony-forming units (CFUs) of candidate bacterial strains that did or did not elicit cytoprotection in the *Drosophila* model, daily for 3 days. Thereafter the experimental groups were subjected to 12 Gy whole-body irradiation, whereupon murine weights were recorded daily. Feeding of vehicle control or lactobacilli strains that did not elicit cytoprotection in the *Drosophila* resulted in similar mortality rates with no statistically significant differences, whereas mice fed either *L. lactis* subsp. *cremoris* or *L. rhamnosus* GG exhibited significantly enhanced preservation of body weight and survival rates compared with vehicle control or non-protective strains (Figures 3A–3D). Indeed, *L. lactis* subsp. *cremoris* protected mice from irradiation-induced mortality to a significantly greater extent than *L. rhamnosus* GG. Intestinal tissue from select candidate strains that have shown similar levels of mortality were histologically examined at 24 h following insult with 12 Gy whole-body irradiation revealed that *L. lactis* subsp. *cremoris* and L. rhamnosus GG mice had significantly fewer apoptotic cells at the base of the colonic crypt compared with vehicle or other control strains of LAB (Figures 3E and 3F). Indeed, apoptotic TUNEL-positive were virtually undetectable at the base of the crypt of the experimental group of mice fed *L. lactis* subsp. *cremoris* and dosed with 12 Gy whole-body (Figures 3E and 3F). Together, these data show that *L. lactis* subsp. *cremoris* elicits potent cytoprotection from radiological damage in the murine colonic tissue.

Lactococcus lactis subsp. *cremoris* ATCC 19257 Protects the Murine Intestine from Dextran Sodium Sulfate-Induced Colitis

In addition to protection against radiological-induced intestinal damage, we used the DSS-induced gut injury and colitis model to assess the efficacy of *L. lactis* subsp. *cremoris* in modulating murine intestinal inflammation. As *L. lactis* subsp. *cremoris* and *L. rhamnosus* GG elicited potent cytoprotection against radiological damage, we focused our experimental efforts on these two beneficial bacteria. Mice were fed 1×10^8 CFU *L. lactis* subsp. *cremoris*, *L. rhamnosus* GG, or vehicle control daily for 14 days. After 14 days, feeding of the bacteria ceased, and 4% DSS was included in the drinking water of the experimental groups. As expected, mice fed vehicle control responded to DSS by exhibiting considerable weight loss, presence of blood in fecal pellets, and high disease activity index (DAI) starting at 4 days following DSS ingestion (Figures 4A and 4B). Mice fed *L. rhamnosus* GG exhibited similar weight loss as vehicle control, and for the most part (except for day 7), exhibited no significant difference in DAI compared with vehicle control (Figures 4A and 4B). Strikingly, mice fed *L. lactis* subsp. *cremoris* exhibited significantly reduced DAI in response to DSS, with mice protected against DSS-induced colitis from day 6 onward (Figures 4A and 4B). Furthermore, mice fed *L. lactis* subsp. *cremoris* exhibited more rapid weight gain after the end of DSS treatment at day 7, i.e., at days 9 and 10 of the experiment (Figure 4A). Examination of colonic tissue at 10 days following onset of DSS treatment confirmed DAI observations in live animals, where *L. lactis* subsp. *cremoris*- (but not *L. rhamnosus* GG)-fed mice exhibited significantly preserved colon length and weight to length ratio compared with vehicle-fed control (Figures 4C and 4D). Furthermore *L. lactis* subsp.

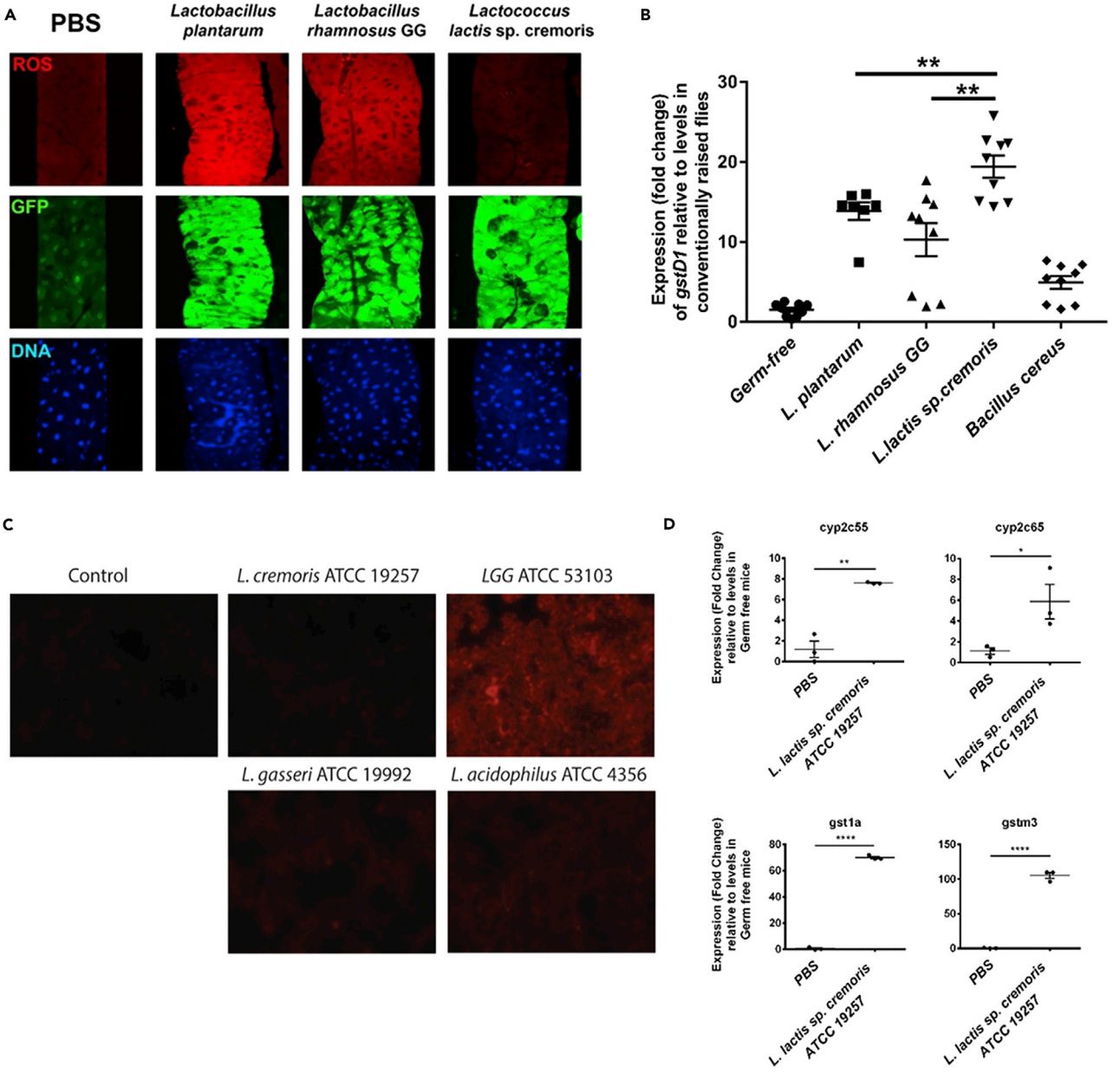

Figure 2. *Lactococcus lactis* subsp. *cremoris* ATCC 19257 Induces the Activation of the Nrf2 Signaling Pathway in the *Drosophila* Intestine by a Mechanism that Does Not Involves the Detectable Generation of Reactive Oxygen Species within Enterocytes

(A) Detection of ROS generation following the ingestion of indicated bacteria by germ-free third instar larvae for 1 h. ROS was detected by oxidation of the hydrocyanine ROS-sensitive dye that is present in the larval food. Shown also is the detection of the Nrf2-responsive anti-oxidant response element-dependent GFP expression in the distal midgut of germ-free gstD1-*gfp* 5-day-old adult *Drosophila* following ingestion of the indicated bacteria.

(B) Detection of transcript enrichment in the midgut of the indicated CncC (Nrf2 in mammals) pathway-responsive genes in germ-free *Drosophila* mono-associated with the indicated bacteria for 4 h. Data are represented as mean ± SEM. Nonparametric unpaired t test **$p < 0.01$, $n = 9$ for all samples except for the *L. plantarum* dataset where $n = 7$.

(C) Detection of ROS generation in cultured cells following contact with the indicated bacteria. Bacterial-induced ROS in cells contacted by the indicated bacteria for up to 30 min. Caco-2 cells seeded in a 96-well format were preloaded with 100 μM hydro-Cy3 and then contacted with 3×10^8/100 μL viable bacteria for the indicated times. Cells were then washed three times with PBS before fluorometric analysis at 575 nm.

(D) Detection of transcript enrichment of CncC pathway-responsive genes in the colonic epithelium of germ-free C57/B6 mice mono-associated with the indicated bacteria for 4 h. Data are represented as mean ± SEM. Nonparametric unpaired t test *$p<0.05$; **$p<0.01$; ****$p<0.0001$, $n = 5$.

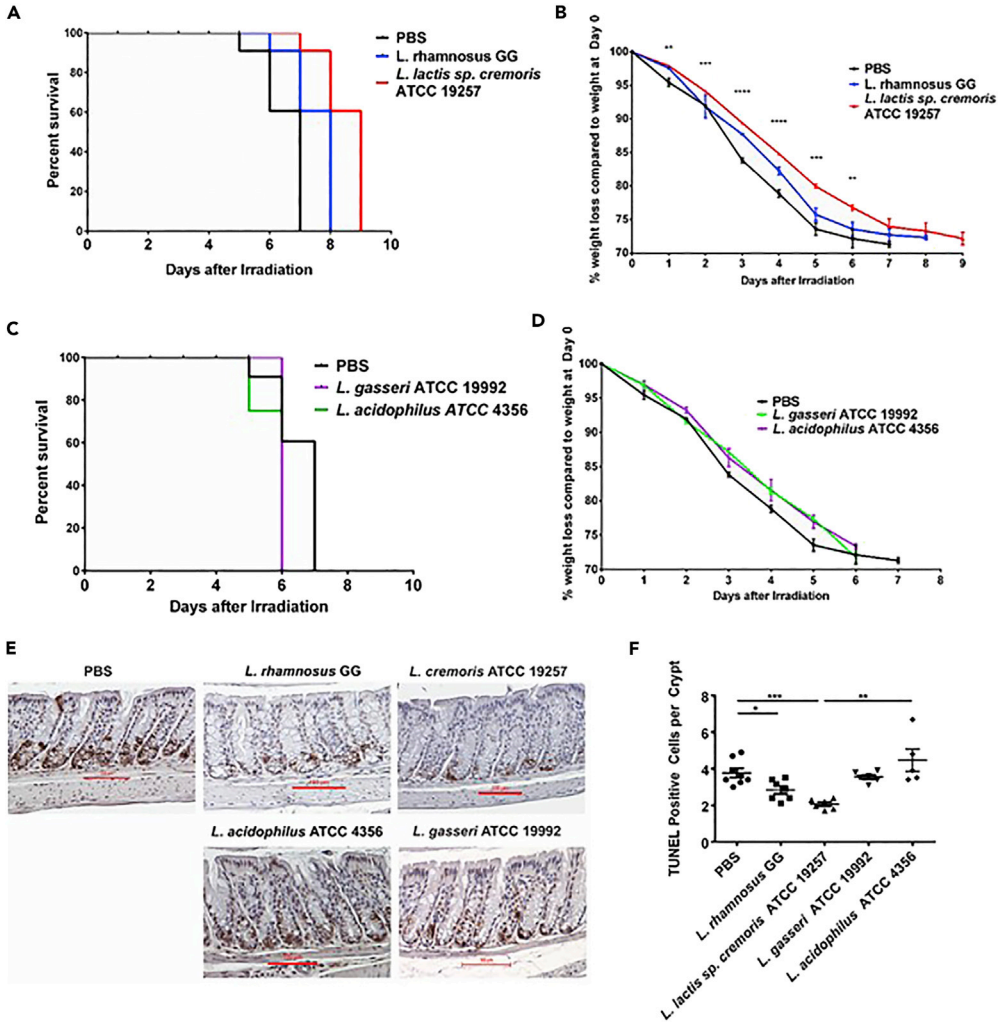

Figure 3. Ingestion of *Lactococcus Lactis* Subsp. *cremoris* Is Cytoprotective Against Radiological Insult in Mice.

A) Survival of 6-week-old C57BL/6 fed the indicated bacteria daily for 3 days followed 12 Gy irradiation insult. Note significantly enhanced survival of only mice treated before irradiation for three days with *L. lactis* subsp. *cremoris* ATCC 19257 (Log-Rank test for untreated vs. *L. lactis* subsp. cremoris treated, P=0.0006, n=5), or with *L. rhamnosus* GG (Log-Rank test for untreated vs. *L. rhamnosus* GG P=0.0202, n=5).

(B) Weight of mice described in (A) measured daily after irradiation insult. Nonparametric unpaired t-test of bacterial *L. lactis* subsp. *cremoris* ATCC 19257 treated against untreated mice. **=P<0.01, ***=P<0.001, ****=P<0.0001, n=5.

(C) Survival of 6-week-old C57BL/6 fed the indicated bacteria daily for 3 days followed 12 Gy irradiation insult. Analysis by Log-Rank test for untreated vs. bacterial treated revealed no significant differences in survival in mice treated *Lactobacillus gasseri* ATCC 19992, or with *Lactobacillus acidophilus* ATCC 4356.

(D) Weight of mice described in (C) measured daily after irradiation insult. Nonparametric unpaired t-test of bacterial treated against untreated mice revealed no significant differences.

(E) Detection of TUNEL-positive cells within colonic tissues harvested from mice described in (A-D). Scale bar size: 100μm.

(F) Quantification of TUNEL-positive cells. Data are represented as mean +/- SEM. Nonparametric unpaired t-test ***p<0.001, n=5.

cremoris-fed mice exhibited markedly improved re-epithelialization and villi tissue morphology compared with vehicle-fed control or *Lactobacillus rhamnosus* GG (LGG) at day 10 of the experiment, with increased numbers of proliferative phospho-histone H3-positive cells, a marker for cell proliferation detectable in intestinal mucosa (Figures 4E and 4F). Finally, we measured the numbers of myeloperoxidase (MPO)-positive cells in the colonic submucosa in these mice. MPO is a major component of neutrophil granulocytes and thus is a faithful marker for the presence of neutrophils in the intestinal tissue. Neutrophils are recruited from general circulation to infected or injured intestinal tissue, including tissue injured in DSS-treated

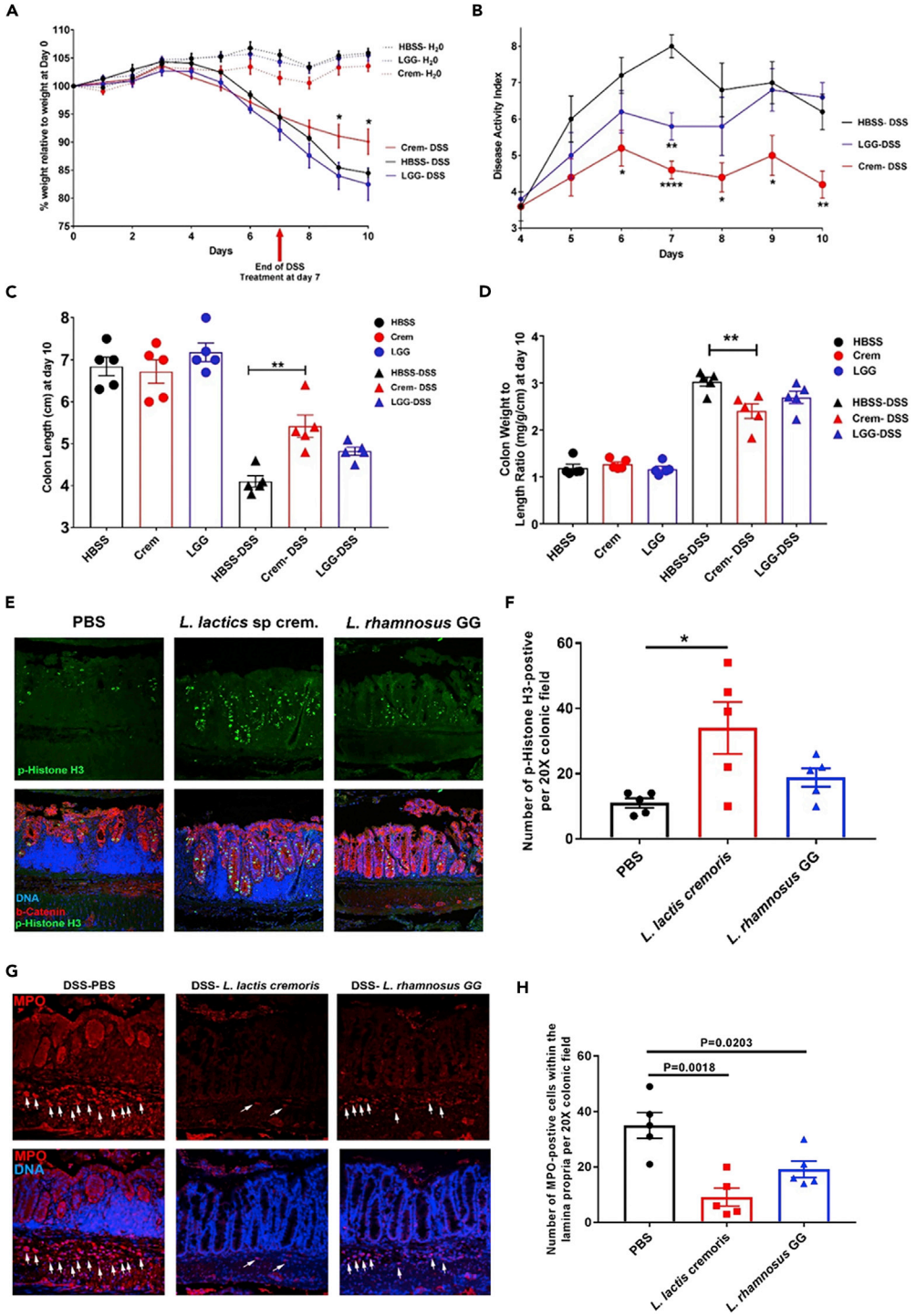

Figure 4. *Lactococcus lactis* subsp. *cremoris* ATCC 19257 Protects the Murine Intestine from Dextran Sodium Sulfate (DSS)-Induced Colitis

(A) Groups of five 6-week-old male C57/B6 mice were administered either 1 × 10^8 CFU total *Lactococcus lactis* subsp. *cremoris* ATCC 19257, or *Lactobacillus rhamnosus* GG, or vehicle control for 14 days. Feeding of bacteria was ceased before 4% DSS being included in the drinking water (day 0) for 7 days in the indicated groups. Control groups of bacteria-fed mice were subjected to regular water. Measurement of weight loss in mice of the aforementioned groups following commencement of the inclusion of 4% DSS in the drinking water. Data are represented as mean ± SEM. Nonparametric unpaired t test *p < 0.05, n = 5.

Figure 4. *Continued*

(B) Disease activity index (DAI) of mice described in (A). Nonparametric unpaired t test *p < 0.05, **p < 0.01, ***p < 0.001. n = 5.

(C) Colon length of mice in (A) and (B) at day 10 following commencement of treatment with DSS. Data are represented as mean ± SEM. Nonparametric unpaired t test **p < 0.01, n = 5.

(D) Colon weight to length ratio of mice in (A) and (B) at day 10 following commencement of treatment with DSS. Data are represented as mean ± SEM. Nonparametric unpaired t test **p < 0.01, n = 5.

(E) Detection of phospho-histone-H3-positive cells in the colonic epithelium of indicated DSS-treated mice at day 10 following commencement of treatment with DSS.

(F) Numeration of phospho-histone-H3-positive cells shown in (E). Data are represented as mean ± SEM. Nonparametric unpaired t test *p < 0.05, n = 5 per group.

(G) Detection of myeloperoxidase (MPO)-expressing cells in the colonic tissue subepithelial compartments of colonic tissue at day 10 following commencement of treatment with DSS. White arrows point to cells stained positive with anti-Myeloperoxidase/MPO antibody that exhibit characteristic polymorphonuclear morphology of neutrophils.

(H) Numeration of MPO-positive cells shown in (G). Data are represented as mean ± SEM. Nonparametric unpaired t test, with p values indicated for each comparison.

mice. *L. lactis* subsp. *cremoris*-fed mice exhibited significantly fewer MPO-positive cells in the colonic submucosa compared with vehicle-fed control or LGG-fed mice at day 10 of the experiment, indicating that inflammation and restitution occurred faster in *L. lactis* subsp. *cremoris*-fed mice (Figures 4G and 4H). Together, these data show that *L. lactis* subsp. *cremoris* potently dampened outward disease symptoms and intestinal tissue damage induced by a DSS gut injury and colitis model in mice.

DISCUSSION

We identify *L. lactis* subsp. *cremoris* ATCC 19257 as a highly efficacious beneficial bacterium, which was successful in producing a desired and intended result of preserving gut tissue health in response to induced inflammation or injury. Importantly, the detected beneficial influences are a specific property of *L. lactis* subsp. *cremoris*, because other strains of LAB tested did not exhibit similar influences in the same injury models. In addition, *L. lactis* subsp. *cremoris* conferred significantly more efficacious influences in dampening intestinal inflammation in the DSS gut injury and colitis model compared with *L. rhamnosus* GG, which is a widely marketed and perhaps the most well-studied beneficial bacterium. To our knowledge, this is the first strain from the *Lactococcus* genus with demonstrated capacity to confer anti-inflammatory properties in the intestine subjected to DSS-induced colitis injury.

Current interventional therapies for intestinal inflammation include oral dosing of 5-aminosalicylic acid, antibiotics, corticosteroids, and immunomodulators and subcutaneous or intravenous administration of biologics (Oliveira and Monteiro, 2017). However, the notion of using bacteria-based platforms in the clinical setting to treat intestinal inflammation is an increasingly recognized viable approach (Xavier, 2016). LAB, mainly from the *Lactobacillus* genus have been reported to suppress inflammation, strengthen gut epithelial barrier function, promote epithelial restitutional responses, and thus offer potential therapy for disorders of the gastrointestinal tract (Derwa et al., 2017; Plaza-Diaz et al., 2017; Vemuri et al., 2017; Pace et al., 2015). Importantly, our identification of *L. lactis* subsp. *cremoris* as a highly efficacious strain in ameliorating gut inflammation, at least in murine models, adds another microbe to the repertoire of beneficial bacteria to treat gut inflammation. Indeed, *L. lactis* subsp. *cremoris* has many key desirable features of beneficial bacteria. *L. lactis* strains are mostly non-colonizing, non-sporulating homolactate facultative anaerobes that have been used extensively in the fermentation of dairy products such as cheese and yoghurt for centuries and therefore fall under the GRAS status. These facts support the likelihood that potential use of *L. lactis* subsp. *cremoris* as a therapeutic bacterium to dampen intestinal inflammation would not be confounded by potential toxicological effects. Clearly, determining the extent to which *L. lactis* subsp. *cremoris* functions similarly in clinical settings as in the murine models is now a matter of investigative priority.

The discovery and demonstration of the highly efficacious nature of *L. lactis* subsp. *cremoris* in preserving tissue health in response to induced gut inflammation also has considerable implications in the search for beneficial microbes. Because other taxonomically related strains of *L. lactis* subsp. *cremoris* do not exhibit beneficial responses, it is likely that the beneficial influences of this stain are due to specific genetic elements and factors harbored within the bacteria. Comparative genomic analysis of taxonomically related beneficial and non-beneficial strains offers a powerful approach toward identifying these bacterial genetic

elements that confer beneficial effects, which is an approach of intense current investigation within our research group. Moreover, identification of such genetic elements would also identify genetic targets within bacteria that may be the basis for a high-throughput screening platform to discover perhaps even more efficacious beneficial microbes. Indeed, employing multiple cell culture and invertebrate platforms to screen candidate beneficial microbes has the potential to generate cumulative evidence and rational for testing the efficacy of candidate beneficial microbes within *in vivo* mammalian models. Screening platforms would include testing if the microbe harbors a desired genetic element, has the capacity to induce nontoxic levels of ROS generation in cultured enterocytes (Jones et al., 2013), or has the capacity to inhibit apoptosis in cultured cell (Lin et al., 2008), to induce epithelial cell movement and would healing *in vitro* (Alam et al., 2014), as well as to confer cytoprotection against oxidative challenge in *Drosophila* (Jones et al., 2015).

Limitations of the Study

The limitations of the current study are that the molecular mechanism whereby *L. lactis* subsp. *cremoris* confers anti-inflammatory influences remains enigmatic. Because we show in Figure 2 that *L. lactis* subsp. *cremoris* ATCC 19257, unlike *L. rhamnosus* GG (Jones et al., 2013), does not induce the cellular generation of ROS, we speculate that *L. lactis* subsp. *cremoris* may induce, or perhaps inhibit, the activation of a distinct set of pro-inflammatory downstream cell signaling pathways. The employment of methodology that measures transcript enrichment in tissues, such as RNA sequencing, or platforms that measure global protein enrichment, such as multiplex ELISA, will be valuable tools to elucidate downstream signaling events in this context. Furthermore, because *L. lactis* subsp. *cremoris* exhibits higher efficacy at ameliorating gut inflammation compared with *L. rhamnosus* GG, and many gut inflammatory states are associated with aggressive and dysregulated activation of innate and adaptive immunity (Cader and Kaser, 2013; Al-Ghadban et al., 2016; Elia et al., 2015), it is also possible that *L. lactis* subsp. *cremoris* may act directly, or indirectly via enterocytes, on immune cell activity in the gut. At any rate, the efficacy of *L. lactis* subsp. *cremoris* at dampening inflammation caused by DSS-induced colitis is highly apparent and merits candidature for use as a bacterial therapy in the clinical environment as well as for the elucidation of the mechanistic mode of action.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods and can be found with this article online at https://doi.org/10.1016/j.isci.2019.01.030.

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AUTHOR CONTRIBUTIONS

R.M.J. and T.M.D. conceived and designed the experiments. L.L. and C.R.N. performed experiments on the *Drosophila* animal model, and T.M.D., B.J.S., B.S.R., and J.A.O. performed experiments on the murine animal model. R.M.J. and T.M.D. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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Supplemental Information

Lactococcus Lactis Subsp. *cremoris* Is an Efficacious Beneficial Bacterium that Limits Tissue Injury in the Intestine

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TRANSPARENT METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
DAPI	Sigma-Aldrich	Cat# 5087410001
anti-Myeloperoxidase/MPO	R&D Systems	Cat# AF3667
Phospho-Histone H3 (Ser10)	Cell Signaling technology	Cat# 9701
β-Catenin (D10A8) XP®	Cell Signaling Technology	Cat# 8480
Bacterial and Virus Strains		
Lactococcus lactis subsp *cremoris*	American Type Tissue Collection	ATCC 19257
Lactobacillus rhamnosus GG	American Type Tissue Collection	ATCC 53103
Lactobacillus gasseri	American Type Tissue Collection	ATCC 19992
Lactobacillus acidophilus	American Type Tissue Collection	ATCC 4356
Lactobacillus plantarum	Isolated in our Laboratory	(Jones et al., 2013)
Bacillus cereus	Isolated in our Laboratory	(Jones et al., 2013)
Chemicals, Peptides, and Recombinant Proteins		
Methyl viologen dichloride	Sigma-Aldrich	Cat# 856177
Phosphate buffered saline	Sigma-Aldrich	Cat# P5493
Paraformaldehyde	Sigma-Aldrich	Cat# 158127
BD Difco™ Lactobacilli MRS Broth	Fisher Scientific	Cat# BD288130
Dextran Sulfate Sodium Salt (36,000-50,000 M.Wt.)	MP Biomedicals	Cat# 02160110
TRIzol™	Invitrogen	Cat# 15596026
Blue Food Color Dye	Spectrum Chemicals	Cat# F1308
Critical Commercial Assays		
QuantiFastSYBR Green PCR master mix	Qiagen	Cat# 15596026
QuantiTect Reverse Transcription Kit	Qiagen	Cat# 205311
RNeasy Kit	Qiagen	Cat# 74106
In Situ Cell Death Detection Kit	Roche Diagnostics Corporation	Cat# 11684795910
ROSstar 550 hydrocyanine probe	LICOR Biotechnology	Cat# 926-20000
ApopTag® Plus Peroxidase In Situ Apoptosis Kit	Millipore Sigma	Cat# S7101
Experimental Models: Cell Lines		
Human: Passage 14-18 HT-29 cells	American Type Tissue Collection	ATCC® HTB-38™
Experimental Models: Organisms/Strains		

Mouse: C57BL/6	Jackson Laboratory	Cat# 000664;RRID:IMSR_JAX:000664
Oligonucleotides		
gstd1F 5'-CTGGTGGACAACGGATTCG-3'	Eurofins Genomics	N/A
rp49F 5'-GCAAGCCCAAGGGTATCGA-3'	Eurofins Genomics	N/A
Cyp2c55-F,5'-GCTGTTGCTATGCTGGTATCT-3'	Eurofins Genomics	N/A
Cyp2c65-F,5'- GAGTTTGCTGGAAGAGGAGTT-3'	Eurofins Genomics	N/A
Gst1a-F, 5'-GGGTGGAGTTTGAAGAGAAGT-3'	Eurofins Genomics	N/A
Gstm3-F, 5'-GTCTGCTGCAGTCCCGATTT-3'	Eurofins Genomics	N/A
β-Actin-F,5'-AATGTGGCTGAGGACTTTGT-3'	Eurofins Genomics	N/A
Software and Algorithms		
Graphpad Prism 6	GraphPad Software	N/A

Bacterial strains and culture preparation

The following bacteria were purchased from the *American Type Culture Collection (ATCC) (Manasas, VA): L. lactis* subsp. *cremoris* ATCC 19257, *Lactobacillus rhamnosus* GG ATCC 53103, *Lactobacillus gasseri* ATCC 19992 and *Lactobacillus acidophilus* ATCC 4356. All media were propagated according to instructions provided by the ATCC. *Lactobacillus plantarum* and *Bacillus cereus* was isolated from the midgut of *Drosophila* within our fly stock collection at Emory University as previously described as previously described (Jones et al., 2013).

***Drosophila* Paraquat resistance assays**

Germ-free *Drosophila* were generated according to our developed protocol (Luo et al., 2016). Whole animal cytoprotection in *Drosophila* was measured in response to Methyl viologen dichloride (Paraquat^{TM}) -induced oxidative stress according to protocol previously outlined (Jones et al., 2015). In this study, Methyl viologen dichloride was purchased from Sigma-Aldrich (Cat# 856177, Lot# STBG9488). Groups of 20 per vial, of 7-day-old adult *Drosophila* of assayed genotypes were starved for 3 hours and then fed a solution of 5% sucrose containing a semi-lethal dose of Paraquat (17.5 mM). This concentration was chosen because it was empirically

tested that for this batch of Methyl viologen dichloride, a concentration of 17.5mM would be lethal to > 90% of germ-free *Drosophila* population within 8 days, or 196 hours. For bacterial mono-association assays, $1x10^{10}$ CFU of pure bacterial cultures were inoculated into sterile vials. To determine that the adult *Drosophila* ingested bacterial monoculture, and that the bacteria remained viable following the introduction of Paraquat, germ-free adult *Drosophila* were fed purified cultures of either *L. lactis* subsp. *cremoris* ATCC 19257, *L. rhamnosus* GG ATCC 53103, *L. gasseri* ATCC 19992 and *L. acidophilus* ATCC 4356, in liquefied *Drosophila* media containing 17.5mM Paraquat. After 96 hours of ingestion of bacterial monoculture and Paraquat, five adult *Drosophila* intestines were dissected without contacting any of the exterior of the fly during dissection, and placed into 1 ml sterile PBS. Thereafter, the CFU of viable bacteria collected per fly intestine was calculated by the plate colony count method. We detected 7.2×10^4 CFU *L. lactis* subsp. *cremoris* ATCC 19257 per adult fly intestine (s.d.=4.2×10^3, *n*=5), and 2.4×10^4 cfu *L. rhamnosus* GG ATCC 53103 per intestine (s.d.=3.6×10^3, *n*=5), 1.4×10^4 CFU *Lactobacillus gasseri* ATCC 19992 per adult fly intestine (s.d.=1.2×10^3, *n*=5), and 9.4×10^3 CFU *Lactobacillus acidophilus* ATCC 4356 per adult fly intestine (s.d.=9.3×10^2, *n*=5). The numbers of bacteria in the intestine of Paraquat-fed flies were comparable to control germ-free flies mono-associated with *L. lactis* subsp. *cremoris* ATCC 19257, and not fed Paraquat, 8.9×10^4 CFU (s.d.=8.7×10^3, *n*=5). Percent surviving flies were recorded and compared by log-rank Martel–Cox test using Graphpad Prism 6 Software (GraphPad Software, Inc. La Jolla, CA). Gut tissue cytoprotection in response to (Paraquat) -induced oxidative stress was analyzed following dissection of the entire alimentary canal, fixing the tissue in 4% paraformaldehyde, followed by TUNEL assay analysis using In Situ Cell Death Detection Kit (Roche Diagnostics Corporation). TUNEL-positive cells were identified by Confocal microscopy.

Assessment of gut permeability in adult *Drosophila* assay

Assessment of gut permeability in adult *Drosophila* was undertaken based on assays developed by Rera et al., 2012 (Rera et al., 2012). Germ-free male *w1118* flies were aged to 5-days old in replicates of twenty. Flies were mono-colonized with $1x10^{10}$ CFU pure cultures of the indicated bacterium for 3 days. Then, flies were subjected to 17.5mM Paraquat solution which induces a leaky gut phenotype within 24 to 48 hours. The paraquat solution was spiked with inert blue food dye. Flies that have a leaky gut are identified by exhibiting a visible blue color within their hemocoel, the primary body cavity of most invertebrates, containing circulatory fluid. Flies were anesthetized daily with CO2 daily and visually scored for the blur dye infiltration and the 'Smurf'-like phenotype. A fly was marked as Smurfed when blue dye had infiltrated thorough the body.

Detection of ROS generation in the germ-free or colonized third instar *Drosophila* larvae

Detection of ROS generation and CncC pathway activation was undertaken as previously described (Jones et al., 2013). Briefly, Drosophila embryos were collected and soaked in 50% bleach for 5 minutes, then washed with sterile PBS, and transferred into a sterile Petri dish containing sterilize *Drosophila* food. Wondering third instar larvae were transferred into another Petri dish containing 2ml liquefied sterile *Drosophila* food containing a total of $1x10^6$ cfu pure bacterial culture, and a final concentration of 100μM of the ROS-sensitive ROSstar 550 *hydrocyanine* probe reagent (LI-COR Biotechnology, Lincoln, NE). After 1 hour, the intestinal tract of third-instar larvae were dissected, fixed in 4% paraformaldehyde, and fluorescence detected by Confocal microscopy.

Animals

All the animal procedures were approved by the Institutional Animal Care and Use Committee of Emory University. All in vivo experiments were carried out in male mice. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). 6-week-old male C57BL6 Germ Free

(GF) mice were purchased from the Emory Gnotobiotic Animal Core (EGAC). EGAC is supported by the Georgia Clinical & Translational Science Alliance and the Emory University School of Medicine**.**

Murine subjects and γ-irradiation

C57BL/6 mice were purchased from Jackson laboratory and maintained the Emory University Department of Animal Resources. For irradiation insults, whole bodies were subjected to 12 Gy of γ-radiation using a γ-Cell 40 ^{137}Cs irradiator at a dose rate of 75 rads/min. Pure cultures of beneficial bacteria (1x10^{8} cfu total) were administered by oral gavage daily for 3 days before irradiation, and body weights and mortality were monitored. Animal experiments were approved by the Emory University institutional ethical committee and performed according to the legal requirements. Histological sections of the colon were prepared from between 5-8 irradiated animals per treatment. Sections were assessed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay (Millipore, Burlington, Massachusetts, USA). TUNEL-positive cells were counted and the average number of positive cells in forty 200× fields per treated animal was determined.

Murine DSS-colitis model

Groups of C57BL/6 mice were purchased from Jackson laboratory and maintained the Emory University Department of Animal Resources. Pure cultures of beneficial bacteria, including *L. rhamnosus* GG, or *L. lactis* cremoris (1x10^{8} cfu total) were administered by oral gavage daily for 14 days. At day 14, Dextran Sodium Sulfate (DSS) was included in the drinking water of some groups of mice to a final concentration of 4%. Murine body weights, and disease activity index were monitored daily until mice had lost 80% body weights (seven days), whereupon DSS was removed from the drinking water. Murine body weights, and disease activity index were

monitored daily for a further three days before mice were sacrificed and histological sections of the colon were prepared for disease activity. All animal experiments were approved by the Emory University institutional ethical committee and performed according to the legal requirements.

Quantitative (q) PCR for Gene Expression Fold Change

RNA from 30 *Drosophila* intestines were mixed and extracted using TRIzol reagent (Invitrogen, Carlsbad, California, USA), and cleaned using RNeasy kit (Qiagen) total RNA clean up protocol. cDNA of these samples was synthesized using QuantiTect Reverse Transcription Kit, and relative transcript levels were measured for each sample in triplicate by quantitative qRT-PCR using QuantiTect SYBR Green PCR Kit (Qiagen). *gstD1* transcripts were amplified using primers gstd1F 5'-CTGGTGGACAACGGATTCG-3', gstD1R 5'-GCCGTACTTCTCCACCAAATACA-3', and samples normalized using primers for the amplification of Rp-49 rp49F 5'-GCAAGCCCAAGGGTATCGA-3', and rp49R 5'-ACCGATGTTGGGCATCAGA-3'. The data generated by qPCR assays were normalized using the average value of the conventionally raised control group. For measurements in mice, dissected colonic tissue was placed in RNA-later. The total mRNA (800 ng) was reverse transcribed into cDNA using QuantiTect® Reverse Transcription Kit (cat # 205311, Qiagen). Relative quantification of mRNA expression was performed using QuantiFast SYBR® Green PCR Kit (cat # 204054, Qiagen) on a MyiQ™ Real time PCR system (Biorad). Delta-delta Ct analysis (ΔΔCT) method was used to quantify relative gene expression compared with Actin controls, using following primers: Cyp2c55-F,5’-GCTGTTGCTATGCTGGTATCT-3’, Cyp2c55-R,5’-ACTGGATTGTGGGAGAATGAA-3’, Cyp2c65-F,5’- GAGTTTGCTGGAAGAGGAGTT-3’, Cyp2c65-R,5’- CGCAGAGTCATGAGTGAGAAG-3’, Gst1a-F, 5’-GGGTGGAGTTTGAAGAGAAGT-3’, Gst1a-R, 5’-TGGCGATGTAGTTGAGAATGG-3’, Gstm3-F, 5’-GTCTGCTGCAGTCCCGATTT-3’, Gstm3-R, 5’-CATAGGTGACCTTGTCCCCTGC-3’, β-

Actin-F,5’-AATGTGGCTGAGGACTTTGT-3’, β-Actin,5’-GGGACTTCCTGTAACCACTTATT-3’.

The data generated by qPCR assays were normalized using the average value of the PBS treatment control group.

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