**Article**

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

**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* activated anti-inflammatory activity in a mouse colitis model
- *L. lactis cremoris* activated the cytoprotective Nrf2 pathway in flies and mice

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**Article**

*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 scarcity of empirical data to substantiate the claims made by 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 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
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 \textit{L. lactis} subsp. \textit{cremoris} resulted in significantly fewer flies with blue dye translocation to the hemolymph than germ-free flies (Figure 1F). In addition, mono-association with \textit{L. rhamnosus} GG resulted in significantly fewer flies with blue dye, albeit to a lesser extent compared with \textit{L. lactis} subsp. \textit{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 \textit{L. lactis} subsp. \textit{cremoris} ATCC 19257, beneficial properties that were markedly more efficacious in the \textit{Drosophila} compared with \textit{L. rhamnosus} GG or other LAB tested.

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

We previously demonstrated that \textit{L. plantarum} contact with cells induces ROS generation in \textit{Drosophila} enterocytes (Jones et al., 2013), and that cellular ROS enzymatically generated in response to contact with \textit{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 \textit{L. lactis} subsp. \textit{cremoris} also induces the generation of ROS in the \textit{Drosophila} midgut cells, thereby postulating a mechanism whereby \textit{L. lactis} subsp. \textit{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 \textit{L. plantarum} and \textit{L. rhamnosus} GG induced high levels of ROS in larval midgut epithelial cells, \textit{L. lactis} subsp. \textit{cremoris} did not induce any detectable amounts of ROS generation in the same cells (Figure 2A). However, we also measured the capability of \textit{L. lactis} subsp. \textit{cremoris} to

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

Table 1. List of Candidate Beneficial Bacteria Used to Mono-Associate Germ-free \textit{Drosophila} and Screened for Their Capacity to Protect \textit{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.
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* < 0.0006, n = 50), or mono-colonized with *Lactobacillus rhamnosus* GG (log rank test for g-f versus g-f + *L. rhamnosus* GG *p* < 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 monoo-associate 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 x 10^6 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 x 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 X 10^9/100 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.
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 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=0.0002, 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.
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 $1 \times 10^8$ 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.
Disease activity index (DAI) of mice described in (A). Nonparametric unpaired t test *p < 0.05, **p < 0.01, ***p < 0.001. n = 5

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.

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.

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.

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.

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.

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 candidate 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.
by increasing the expression levels of genes

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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* | ATCC 19257 |
| *Lactobacillus rhamnosus* GG | ATCC 53103 |
| *Lactobacillus gasseri* | ATCC 19992 |
| *Lactobacillus acidophilus* | 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** |        |            |
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™)-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 \times 10^4 CFU L. lactis subsp. cremoris ATCC 19257 per adult fly intestine (s.d.=4.2 \times 10^3, n=5), and 2.4 \times 10^4 cfu L. rhamnosus GG ATCC 53103 per intestine (s.d.=3.6 \times 10^3, n=5), 1.4 \times 10^4 CFU Lactobacillus gasseri ATCC 19992 per adult fly intestine (s.d.=1.2 \times 10^3, n=5), and 9.4 \times 10^3 CFU Lactobacillus acidophilus ATCC 4356 per adult fly intestine (s.d.=9.3 \times 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 \times 10^4 CFU (s.d.=8.7 \times 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
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'-CTGGTGGAACAACGGATTCG-3', gstd1R 5'-GCCGTACTTCTCCACAAATACA-3', and samples normalized using primers for the amplification of Rp-49 rp49F 5'-GCAAGCCCAAGGGGTATCGA-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'-GCTGTGTCTATGCTGTCT-3', Cyp2c55-R, 5'-ACTGGATTTGAGGAATGAA-3', Cyp2c65-F, 5'-GAGTTTGCTGGGAAGAGGAGTT-3', Cyp2c65-R, 5'-CGCAGAGTCATGGAGAGAG-3', Gst1a-F, 5'-GGGTGGAGTTTGAAGAGAAGT-3', Gst1a-R, 5'-TGGCGATGTAGTGGAGGAATGG-3', Gstm3-F, 5'-GTCTGCTGAGTCCCGATTT-3', Gstm3-R, 5'-CATAGGTGACCTGTGCCCTGC-3', β-
Actin-F, 5'-AATGTGGCTGAGGACTTTGT-3', β-Actin, 5'-GGGACTTCCTGTAACCTATT-3'.

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

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