Ectopic Expression of Innate Immune Protein, Lipocalin-2, in *Lactococcus lactis* Protects against Gut and Environmental Stressors

Piu Saha, PhD[1], **Benoit Chassaing, PhD**[2], **Beng San Yeoh, BS**[1], **Emilie Viennois, PhD**[2], **Xia Xiao, BS**[1], **Mary J. Kennett, PhD**[3], **Vishal Singh, PhD**[1], and **Matam Vijay-Kumar, PhD**[1,4,*]

[1]Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA 16802, USA

[2]Center for Inflammation, Immunity & Infection, Institute for Biomedical Sciences, Georgia State University, Atlanta, GA 30303, USA

[3]Departments of Veterinary and Biomedical Sciences, The Pennsylvania State University, University Park, PA 16802, USA

[4]Department of Medicine, The Pennsylvania State University Medical Center, Hershey, PA 17033, USA

Abstract

Background—Lipocalin 2 (Lcn2) is a multifunctional innate immune protein that exhibits antimicrobial activity by the sequestration of bacterial siderophores, regulates iron homeostasis, and augments cellular tolerance to oxidative stress. Studies in the murine model of colitis have demonstrated that Lcn2 deficiency exacerbates colitogenesis, however, the therapeutic potential of Lcn2 supplementation has yet to be elucidated. In light of its potential mucoprotective functions, we herein investigated whether expression of Lcn2 in the probiotic bacterium can be exploited to alleviate experimental colitis.

Methods—Murine Lcn2 was cloned into the pT1NX plasmid and transformed into *Lactococcus lactis* to generate *L. lactis* expressing Lcn2 (Lactis^Lcn2) or the empty plasmid (Lactis^Con). Lactis^Lcn2 was characterized by immunoblot and ELISA, and tested for its antimicrobial efficacy on *Escherichia coli*. The capacity of Lactis^Lcn2 and Lactis^Con to withstand adverse conditions were tested using *in vitro* viability assays. Dextran sodium sulfate colitis model was employed to investigate the colonization ability and therapeutic potential of Lactis^Lcn2 and Lactis^Con.

Results—Lcn2-derived from Lactis^Lcn2 inhibited the growth of *E. coli* and reduced the bioactivity of enterobactin (*E. coli*-derived siderophore) *in vitro*. Lactis^Lcn2 displayed enhanced

tolerance to adverse pH, high concentration of bile acids and oxidative stress *in vitro*, and survived better in the inflamed gut than Lactis[Con]. Consistent with these features, Lactis[Lcn2] displayed better mucoprotection against intestinal inflammation than Lactis[Con] when administered into mice with DSS-induced acute colitis.

Conclusions—Our findings suggest that Lcn2 expression can be exploited to enhance the survivability of probiotic bacteria during inflammation, which could further improve its efficacy to treat experimental colitis.

Keywords

Introduction

Inflammatory bowel diseases (IBD) are idiopathic disorders of the gastrointestinal tract that are characterized by chronic relapsing intestinal inflammation. IBD, such as Crohn's disease (CD) and ulcerative colitis (UC), are associated with the upregulation of many acute-phase proteins (APPs; i.e., C-reactive protein, serum amyloid A, sIL-1Ra). The exploration of the biologic activities of APP has resulted in the development of biological therapeutics to treat IBD[1]. However, currently, there are no universally acceptable route by which these therapeutics can be administered efficiently. Moreover, the therapeutic potential of one of the most dynamically upregulated APP, Lipocalin 2 (Lcn2; aka Neutrophil Gelatinase-Associated Lipocalin or NGAL, siderocalin, 24p3) is considerably unknown[2, 3].

Lcn2 is a small 25 kDa protein that is secreted by a variety of immune and non-immune cells, particularly in response to inflammation[2]. The levels of circulating and mucosal Lcn2 are reported to increase by log orders of magnitude in various inflammatory conditions including IBD[3–9]. Previously, we have shown that fecal Lcn2 is a dynamic, sensitive and non-invasive biomarker of gut inflammation[3]. Among its diverse physiological functions, one well-established role of Lcn2 is to sequester bacterial siderophores, such as enterobactin (Ent) and thus limit the growth of iron-dependent bacteria[4, 10–12]. In addition, Lcn2 also plays a role in facilitating the hypoferremia of inflammation and suppressing the generation of catalytic iron[12]. Such mechanisms suggest that the upregulation of Lcn2 during intestinal or systemic inflammation may mediate alterations in the gut microbiota, particularly during IBD[13–15]. While Lcn2 deficiency has been shown to potentiate gut dysbiosis in mice and increase their susceptibility to colitis[16, 17], the therapeutic potential of Lcn2 supplementation has yet to be elucidated.

Numerous therapeutic molecules have been developed to treat experimental colitis model, however, their utility is impeded by the high cost associated with long-term treatment. Such constraint could be potentially circumvented by employing a recombinant lactic acid bacteria to deliver therapeutic biologicals[18]. The use of bacterial vector would allow for a more cost-effective and direct delivery of therapeutics to the site of inflammation and avoid the risk of systemic side effects. *Lactococcus lactis* genetically modified to secrete the anti-inflammatory cytokine IL-10 is perhaps the most promising application of this strategy and had been demonstrated in several studies to be effective in treating gastrointestinal

inflammation[19, 20]. Subsequent studies also elucidated the efficacy of various recombinant *L. lactis* in mitigating experimental colitis, including *L. lactis* bioengineered to provide mucosal delivery of IL-27[21], TGF-β1, elafin, secretory leukocyte protease inhibitor (SLPI)[22], and anti-TNF nanobodies[23]. Despite the feasibility of using *L. lactis* as a mucosal delivery vector, the efficacy of recombinant *L. lactis* is still critically impacted by its inherent deficiencies to survive in the inflamed gut. Accordingly, we sought to provide evidence that the fitness of *L. lactis* could be further modified as an innovative strategy to improve its efficacy to treat experimental colitis.

In this study, we investigated the therapeutic potential of Lcn2 expression in augmenting the persistence and colonization of a probiotic bacterium in the inflamed gut. We demonstrated that ectopic expression of Lcn2 in *L. lactis* substantially augments its capacity to withstand a spectrum of environmental stressors, including adverse pH, bile acids, and H_2O_2-induced oxidative stress. We confirmed that *L. lactis*-secreted Lcn2 is bioactive in sequestering Ent (a bacterial siderophore that is essential for *E. coli* iron acquisition) and thus able to inhibit the growth of *E. coli in vitro*. By employing the murine model of dextran sulfate sodium (DSS)-induced acute colitis, we provide evidence that Lcn2 expression confers a survival advantage to *L. lactis* and allows it to robustly colonize the inflamed gut than *L. lactis* harboring the empty vector. Further, we demonstrate that Lcn2-expressing *L. lactis* is more effective than control *L. lactis* in mediating mucoprotection to colitic mice. Collectively, our results highlight that genetic modification of probiotic bacteria to produce and secrete Lcn2 may offer a feasible strategy to improve the stability, retention, and efficacy of probiotic bacteria in treating experimental colitis.

Materials and Methods

Reagents

Reagent grade dextran sulfate sodium salt (DSS, M.W. 36–50 kDa, Ref = 160110) was purchased from MP Biomedicals (Solon, OH). Duoset ELISA kits for mouse Lcn2 and keratinocyte-derived chemokine CXCL1 (KC), serum amyloid A (SAA) and biotinylated murine Lcn2 antibody were obtained from R&D Systems (Minneapolis, MN). Hydrogen peroxide (H_2O_2), myeloperoxidase (MPO), hexadecyl trimethyl ammonium bromide, RNAlater®, TRI Reagent®, iron-free enterobactin (Ent), dimethyl sulfoxide, glucose, sodium taurocholate, glycocholate, lithocholate and deoxycholate were procured from Sigma-Aldrich (St. Louis, MO). Guaiacol (2-methoxyphenol) were obtained from Alfa Aesar (Ward Hill, MA). Erythromycin was purchased from Amresco (Solon, OH). The iron atomic absorption (AA) standard was purchased from RICCA Chemical Company. SYBR® Green mix and qScript cDNA synthesis kit were procured from Quanta Biosciences. Mouse recombinant (rec)-Lcn2 (free from endotoxin, siderophore, and iron) was obtained from Cell Signaling. All other chemicals used in the present study were reagent grade and procured from Sigma.

Mice

C57BL/6 WT mice were maintained and bred under specific-pathogen-free conditions in the animal house facility at Pennsylvania State University, PA. Mice were housed in cages (max.

5 mice per cage) containing sawdust bedding and paper tissue nestlets, and fed on chow-control diet *ad libitum* with unrestricted access to water throughout the study. The cages were kept at 23°C and underwent a 12 h light/dark phase cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Pennsylvania State University.

Bacteria

Lactococcus lactis strain MG1363 was grown in M17 broth or agar (1.5% [wt. /vol]) media supplemented with 0.5% glucose and incubated overnight under anaerobic conditions at 30°C. Recombinant *L. lactis* strains were selected in M17 with 0.5% glucose in the presence of 5.0 μg/ml erythromycin (henceforth described as selective M17). K12 *E. coli* strain was acquired from the *E. coli* Genetic Stock Center, Yale University. Ent-overexpressing *E.coli* isogenic mutant *ferrienterobactin permease* (fepA) was gifted from Dr. Kathleen Postle, Pennsylvania State University. Non-pathogenic WT K12 *E. coli* and fepA were grown in LB media (Sigma) containing kanamycin (50 μg/ml) overnight at 37°C. The bacterial colony-forming unit (CFU) were adjusted based on the optical density at 600 nm.

Construction of recombinant bacterial strains

Murine Lcn2 cDNA (Sino Biological Inc.) was amplified using primers Lcn2-Spe1 5'-GGACTAGTCCTCAGTTGTCAAGCATTGGTCGG-3' and Lcn2-BamH1 5'-CGCGGATCCGCGACCATGGCCCTGAGTGTCA-3'. Amplicon and plasmid pT1NX (LMBP 3498; available from the BCCM/LMBP Plasmid Collection, Ghent University, Belgium (http://bccm.belspo.be/about-us/bccm-lmbp) were then digested by restriction enzymes BamH1 and Spe1 (Roche) to generate compatible ends. Lcn2 transgene was ligated (T4 DNA ligase, Roche) into pT1NX plasmid at BamH1/Spe1 site. Recombinant plasmids were electroporated into competent *L. lactis* strain MG1363, which was further plated on erythromycin-containing M17 plates for positive selection. Recombinant *L. lactis* was verified via PCR using primers Lcn2-start 5'GTCCTGGGCCTGGCTCTG3' and pT1NX-start AGTCATACTTTCTGCTGCAG (targets upstream end of Lcn2 transgene), and Lcn2-stop CTCTGGGCCTCAAGGACG and pT1NX-stop TTTCGGGCTTTGTTAGCAGC (targets downstream end of Lcn2 transgene). Plasmid was further purified from isolated clones, and transgene presence was verified by BamH1/Spe1 digestion and agarose gel electrophoresis.

Characterization of Lcn2 expression in *L. lactis*

Recombinant Lcn2-expressing *L. lactis* (Lactis[Lcn2]) and *L. lactis* transformed with empty plasmid (Lactis[Con]) were cultured in selective M17 media as described above for 24 and 48 h. Cultures were centrifuged at 10,000 *g* for 5 min, and the cell supernatant and pellet were collected. The supernatant was treated with 100 μl of 100% trichloroacetic acid (TCA) and incubated for 10 min on ice to precipitate proteins. The supernatant protein precipitate and cell pellet were resuspended in RIPA buffer (Cell Signaling) containing protease inhibitor cocktail (Roche). Western blotting was performed by standard methods using 4–20% gel (Bio-Rad) and murine Lcn2 polyclonal antibody. The concentration of secreted Lcn2 was quantitated using Lcn2 Duoset ELISA kit according to the manufacturer's protocol. Cell

lysates and culture supernatant of *L. lactis* transformed with empty plasmid (referred as Lactis[Con]) were used as controls.

Sample preparation, labeling and two-dimensional gel electrophoresis

Protein lysates were obtained from Lactis[Lcn2] and Lactis[Con]. Briefly, Lactis[Lcn2] and Lactis[Con] were pelleted and proteins were extracted by sonication in a lysis buffer (0.5 mM EDTA, 50 mM Tris-HCl, 50 mM NaCl, 1 mM DTT, 5% Glycerol). Protein samples were prepared using 2D-clean-up for subsequent 2D analysis. For 2D-clean-up, one hundred micrograms of proteins were precipitated, purified and cleaned by a 2D-clean-up kit (GE Healthcare) according to manufacturer protocol. Pellets of precipitated proteins were resuspended in 60 μl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 25 mM Tris-HCl pH 8.8). Twenty-five micrograms of Lactis[Lcn2] or Lactis[Con] samples were labeled with 200 pmol N-hydroxy-succinimidyl ester of cyanine dye Cy3 and with 200 pmol N-hydroxy-succinimidyl ester of cyanine dye Cy5, respectively (GE Healthcare Life Science, Piscataway, NJ). After quenching with 10 mM lysine, the labeled proteins were mixed. Sample buffer (7 M urea, 4 M thiourea, 4% CHAPS, 2% DTT, 2% IPG buffer, pH 4-11 NL (GE Healthcare Life Science, Piscataway, NJ) and Rehydration Solution (7 M urea, 4 M thiourea, 4% CHAPS, 1% DTT, 1% IPG) were added to a final volume of 350 μl for each gel. First-dimension Iso Electric Focusing (IEF) was performed using 24-cm IPG strips (pH 4–7, GE Healthcare Life Science, Piscataway, NJ) in Ettan IPGphor (GE Healthcare Life Science, Piscataway, NJ). After IEF, the strips were equilibrated, reduced, alkylated and stained by sequential incubation in 1.5% DTT equilibration buffer (50 mM Tris–HCl, pH 8.8), 6 M urea, 30% glycerol, and 2% SDS) and 4.5% iodoacetamide equilibration buffer slightly colored with bromophenol blue for 20 min each. The second-dimension SDS-polyacrylamide gel electrophoresis was conducted on a 10% polyacrylamide gel in the Ettan DALT II system separation unit (GE Healthcare Life Science, Piscataway, NJ) until the tracking dye reached the bottom of the gel. After completion of two-dimensional electrophoresis, gel images were acquired on a Typhoon Trio (GE Healthcare) at appropriate wavelengths for Cy3 and Cy5 dyes and analyzed using the DeCyder image analysis software (v. 7.0, GE Healthcare Life Science, Piscataway, NJ).

Quantification of bacterial growth

Overnight-grown recombinant Lactis[Lcn2] and Lactis[Con] (1×10^6 CFU) were placed into selective M17 media prepared at different pH (4.0–8.0) or in the presence of primary bile salts (taurocholate or glycocholate; 0–100 mM) or secondary bile salts (lithocholate and deoxycholate; 0–0.1 mM) and then incubated under anaerobic conditions at 30°C for 5 h. Optical density was measured every hour to assess bacterial growth at 600 nm.

Measurement of bacterial viability in the presence of hydrogen peroxide

Overnight-grown recombinant Lactis[Lcn2] and Lactis[Con] (1.0×10^6 CFU) were incubated with H_2O_2 (0–10 mM) for 5 h in selective M17 media under aerobic or anaerobic conditions at 30°C. Viable cells were determined by comparing OD_{600} of H_2O_2-treated and non-treated in each 1 h time point.

In vitro myeloperoxidase (MPO)-mediated bacterial killing assay

The MPO-mediated bacterial killing assay was performed as described by Atosuo and Lilius[24]. Briefly, the reaction mixture was prepared by first adding MPO (5.0 μg/ml) in sterile PBS. H_2O_2 (30 μM) was next added and incubated at room temperature for 5 min. Finally, overnight-grown recombinant Lactis[Lcn2] or Lactis[Con] ($1.0x10^6$ CFU) were added to the reaction mixture. After 5 h of incubation at 30°C, the reaction mixture was serially diluted and plated on selective M17 agar plates in triplicates. Plates were incubated overnight at 30°C, and the bacterial CFU were quantified.

Determination of bacterial iron content

The cellular iron content was measured in bacterial lysates from overnight-grown Lactis[Lcn2] and Lactis[Con] cultures [25]. Briefly, an equal amount of protein lysates (Lactis[Lcn2] or Lactis[Con]) were mixed with an equivalent volume of solution containing hydrochloric acid (0.1 mg/ml), trichloroacetic acid (1.0 mol/l) and thioglycolic acid (30 ml/l) to precipitate proteins and released protein-bound iron. After centrifugation at room temperature (6,200 g for 15 min), the supernatants were collected and mixed with an equal volume of chromogen solution containing 1.5 M ferrozine and 1.5 M sodium acetate. The optical density of the chromogen was measured at 562 nm. Total iron levels were estimated using a standard curve generated with the iron AA standard (0–5 μg/ml).

MPO inhibition assay

Bacterial cell lysates (Lactis[Lcn2] and Lactis[Con], 5.0 μg of protein) and Ent (10 μM) were pre-incubated with MPO (100 mU, final concentration) for 5 min. The MPO reaction was initiated by adding final concentrations of 100 mM guaiacol and $6.7x10^{-3}$ % H_2O_2. The change in absorbance at 470 nm was measured over a period of 10 min at 1 min intervals. MPO activity determined via guaiacol method was calculated as previously described[26].

E. coli K12 growth inhibition assay

E. coli K12 strain MG1655 ($1x10^6$ CFU) were grown in the presence or absence of bacteria lysates (Lactis[Lcn2] or Lactis[Con], 5.0 μg of protein) for 5 h in LB media at 37°C. Bacterial growth was determined by measuring OD_{600} every hour.

Chrome Azurol S (CAS) assay

CAS agar plates were prepared according to the protocol as described previously[27]. Overnight bacterial cultures grown in LB media were placed on CAS agar plates at indicated concentrations in the absence or presence of Lactis[Lcn2] or Lactis[Con], incubated overnight at 37°C and monitored for orange-coloured halo formation. CAS gives a unique blue color when in complex with iron but turns orange when bacterial siderophores (e.g., Ent) chelates the iron from CAS. The intensity of orange halo formation is directly proportional to the concentration of siderophores secreted by the live bacteria.

DSS-induced colitis model

Female WT mice (6–8 weeks old, n=6) were administered 1.5% DSS in drinking water to induce acute colitis as described previously[28]. Briefly, mice were treated with one cycle of

1.5% DSS (MP Biological) in drinking water for 7 days followed by regular water for 4 days. Induction of colonic inflammation was confirmed via fecal occult blood, diarrhea and loss in body weight. For the therapeutic study, mice were administered with Lactis[Lcn2] or Lactis[Con] (1.0×10^8 CFU/mouse) via oral gavage once on the following days upon withdrawal of DSS treatment. After 4 days of bacteria administration, mice were euthanized and severity of colitis was analyzed.

Euthanasia and blood collection

At the termination of the experiment, mice were euthanized via CO_2 asphyxiation and analyzed for standard colitis parameters as described previously [29]. Blood was collected at the time of euthanasia in BD microtainer® (Becton, Dickinson, Franklin Lakes, NJ), via retro-orbital plexus. Hemolysis-free sera were obtained after centrifugation and stored at −80°C until further used.

Bacterial quantification in fecal samples, cecal content, and colonic mucosa

Fecal samples from Lactis[Lcn2] or Lactis[Con]-treated colitic mice were collected at 48, 72 and 96 h post-inoculation. Cecal content and colonic tissue were collected on the day of euthanasia and homogenized in sterile PBS (100 mg/ml). The fecal, cecal and colon homogenates were centrifuged at 200 rpm for 1 min and plated (100 μl) on selective M17 agar plates. Plates were incubated overnight at 30°C and bacterial CFU were quantified.

Colonic MPO assay

MPO assay was performed according to the previously described method[26]. Briefly, frozen or fresh colon tissue (50 mg) was homogenized in 1 mL of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethyl ammonium bromide, freeze-thawed 3x, sonicated, centrifuged (10000 g, 4°C) and the clear supernatants were collected. The reaction was initiated by adding final concentrations of 50 mM guaiacol and 0.002% H_2O_2 to the clear supernatant prepared in a 96-well plate (Corning). The change in absorbance at 470 nm was measured over a period of 10 min at 1-min intervals. One unit of MPO activity is defined as the amount that increases absorbance at 470 nm by OD of 1.0 per min at 25°C, calculated from the initial rate of reaction.

Enzyme-linked immunosorbent assay (ELISA)

Serum KC and SAA were measured by ELISA kits according to the manufacturer's protocols. Frozen or freshly collected feces were reconstituted (100 mg/mL) in PBS containing 0.1% Tween 20, vortexed (30 min, room temperature) and centrifuged (10,000 g, 4°C) to collect clear supernatants for fecal Lcn2 quantification as described previously[3].

Quantitative RT-PCR

Mouse distal colons were collected in RNAlater®. Total colonic RNA was extracted using TRI Reagent® as per manufacturer's protocol. Purified RNA was used to synthesize cDNA for qRT-PCR using SYBR® Green mix according to the manufacturer's protocol. qRT-PCR was performed in StepOnePlus[TM] real-time PCR instrument (Life Technologies, Grand Island, NY). Sequences of primers used for qRT-PCR were (sense and antisense

respectively): *KC* 5'-TTGTGCGAAAAGAAGTGCAG-3', and reverse, 5'-TACAAACACAGCCTCCCACA-3'; *TNFa* 5'-ACTCCAGGCGGTGCCTATGT-3' and 5'-AGTGTGAGGGTCTGGGCCAT-3'; *iNOS* 5'-TTTGCTTCCATGCTAATGC-GAAAG-3', and 5'-GCTCTGTTGAGGTCTAAAGGCTCCG-3'; *36B4* 5'-TCCAGGCTTTGGGCATCA and 5'-CTTTATTCAGCTGCACATCACTCAGA-3'. Thermal profile for the reaction was: initial denaturation at 95°C for 3 min and 40 cycles of denaturation (95°C for 15 s) and annealing and extension (60°C for 1 min). Relative fold difference between groups was calculated using comparative Ct ($2^{--\ -Ct}$) method. Results obtained were normalized with the housekeeping *36B4* gene.

Histology

Swiss rolls of colons were fixed in 10% buffered formalin (Fisher Scientific) overnight and then stored in 70% ethanol. Paraffin embedding tissue sections (5 microns) were stained with hematoxylin & eosin (H&E) using standard protocols at the Animal Diagnostics Laboratories, Pennsylvania State University. To detect the presence of acidic mucin in the goblet cells, alcian blue staining (pH 2.5) was performed in 5 μm section of a Swiss roll made from the colon using Alcian blue (pH 2.5) stain kit from Vector laboratories, USA. Histological scoring for colonic inflammation was graded as described previously[30, 31] based on intestinal lesions, inflammation, mucosa, and percentage of affected area.

Statistical analysis

All experimental results were reproduced in at least three independent experiments performed in triplicate. All values in the results are expressed as mean ± SEM. Statistical significance between two groups was analyzed using unpaired, two-tailed t-test. Data from more than two groups was compared using a one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test (when to compare the mean of each column with the mean control column) or Tukey's multiple comparison tests (when to compare the mean of each column with the mean of every other column). $p < 0.05$ was considered as statistically significant. All statistical analyses were performed with the GraphPad Prism 7.0 program (GraphPad, Inc, La Jolla, CA).

Results

Generation *L. lactis* producing Lipocalin 2

Overexpression of Lcn2 has been demonstrated to enhance cellular resistance to oxidative stress and apoptosis in various mammalian cell lines[32–36]. To investigate whether Lcn2 could be exploited to augment the stability of probiotic bacteria, we have engineered recombinant *L. lactis* strain expressing either the murine Lcn2 (Lactis[Lcn2]) or the empty plasmid as control (Lactis[Con]). The pT1NX plasmid employed herein is a well-characterized secretion vector for *L. lactis*. Accordingly, we inserted the Lcn2 gene into the pT1NX plasmid at the *BamH1/Spe1* site downstream of the P1 promoter and USP45 signal sequence (Fig. 1A). Construction of Lcn2 under the P1 promoter allows for constitutive expression, whereas USP45 encodes for the sequence signal to drive the secretion of Lcn2 from *L. lactis*. In addition, pT1NX plasmid contains the erythromycin resistance gene, which allowed positive selection for Lactis[Lcn2] and Lactis[Con].

We verified that the growth curves of Lactis[Lcn2] and Lactis[Con] were comparable, which indicate that the expression of Lcn2 did not impede the growth of *L. lactis* (Fig. 1B). Immunoblot of Lactis[Lcn2] cell lysate confirmed the expression of Lcn2 as ~25 and 50 kDa bands (Fig. 1C) which corresponded to the monomeric and dimeric forms of Lcn2[37, 38], respectively. While the levels of intracellular Lcn2 in Lactis[Lcn2] remained unchanged from 24 to 48 h of culture (Fig 1D), they were higher in the culture supernatant at 48 h than at 24 h (Fig 1E), suggesting that Lcn2 accumulated extracellularly over time upon secretion, or released from lysed bacteria. The appearance of Lcn2 band from Lactis[Lcn2] with molecular weight lesser than 25 kDa could be due to the lack of post-translational modification, such as glycosylation, in bacteria. The analysis of Lactis[Lcn2] and Lactis[Con] cell lysates, respectively labeled with Cy5 and Cy3 by 2D differential gel electrophoresis, demonstrated that the ectopic expression of Lcn2 did not alter the overall proteome expression in Lactis[Lcn2] (Fig 1F). The loss of the 50 kDa band in the 2D gel could be due to the use of DTT and iodoacetamide, which are strong reducing and alkylating agents, respectively, that reduce and prevent the formation of disulfide bonds in the sample preparation. In comparison, the 50 kDa band observed in the immunoblot could be potentially explained by the use of β-mercaptoethanol, which is relatively less efficient in ensuring complete removal of protein dimers.

Recombinant Lcn2 expressed by *L. lactis* is bioactive

Among its well-characterized functions, Lcn2 is known to sequester Ent from *E. coli*, thus preventing their acquisition of iron and inhibiting their growth. Hence, we first investigated the bioactivity of Lactis[Lcn2]-derived Lcn2 by assessing its antimicrobial and Ent-sequestering properties. Indeed, the cell lysates from Lactis[Lcn2], but not Lactis[Con], significantly inhibited the growth of *E. coli* K12 (MG1655; Lcn2-sensitive strain, Fig. 2A). We next assessed the ability of Ent to inhibit neutrophil myeloperoxidase (MPO) in the presence of Lactis[Lcn2] cell lysate. Previously, we have shown that Ent can inhibit the MPO activity by interfering the peroxidase-catalyzed reaction; Ent interferes with compound I [oxoiron, Fe(IV)O] and reverts the enzyme back to its native ferric [Fe(III)] state[26]. Consistent with our previous report[26], 10 μM of Ent inhibited MPO activity by approximately 60% (Fig. 2B). However, the addition of cell lysate from Lactis[Lcn2], but not Lactis[Con], rescued the MPO activity from Ent-mediated inhibition (~10% inhibition; Fig. 2B), thus implicating that Lactis[Lcn2]-derived Lcn2 is bioactive in sequestering Ent and neutralize its bioactivity.

To verify these findings, we co-cultured the recombinant *L. lactis* with Ent-overproducing *E. coli* (ΔfepA mutant; K12) on chrome azurol S (CAS) media that detects siderophore production. The formation of orange halo due to iron-chelating Ent from ΔfepA *E. coli* was not affected when co-cultured with the control Lactis[Con]. However, the presence of Lactis[Lcn2] dose-dependently inhibited the size of the halo formed by ΔfepA *E. coli,* which further confirms the bioactivity of Lactis[Lcn2]-derived Lcn2 (Fig. 2C).

Intriguingly, we observed that Lactis[Lcn2] on CAS media could also form a halo, indicative of iron-binding property even though *L. lactis* is not known to produce any siderophore[39, 40] (Fig. 2C). This observation correlates with the approximately 100-fold increase in

intracellular iron levels in Lactis^{Lcn2} compared with Lactis^{Con} (Fig 2D). Since Lactis^{Lcn2} lacks the mechanism to uptake the secreted Lcn2, therefore, it is unlikely that Lcn2 could facilitate iron intake. Instead, we envisioned that the cytosolic Lcn2 might have trapped the iron in the cell, thus resulting in the accumulation of cellular iron. The ability of Lactis^{Lcn2} to sequester and possibly deplete iron bioavailability may further explain their capability to inhibit the growth of *E. coli in vitro*.

Lcn2 expressing *L. lactis* displays enhanced tolerance to environmental stressors

The efficacy of probiotic bacteria can be significantly mitigated by deficiencies to withstand the harsh environmental stressors in the gut, especially the inflammatory environment encountered during IBD. Several studies have reported that Lcn2 overexpression in various mammalian cell lines could enhance their cellular resistance to oxidative stress[34, 35]. Hence, we sought to investigate whether expression of Lcn2 would confer any advantage to Lactis^{Lcn2} against adverse conditions. Alteration in extracellular pH, for instance, could induce cellular stress and impede bacterial growth[41]. While we observed Lactis^{Con} to be sensitive to pH changes, we noted that Lactis^{Lcn2} could grow more robustly over a range of physiological pH levels (pH 5–8; Fig. 3A) *in vitro*. The growth advantage of Lactis^{Lcn2} over Lactis^{Con} at adverse pH could be possibly due to reduced pH-mediated stress in the presence of Lcn2.

Aside from pH changes, bacteria traversing the gastrointestinal tract could be exposed to primary bile salts (i.e. taurocholate and glycocholate) that are toxic to most bacteria [42]. Indeed, 0.001 mM of taurocholate and glycocholate were sufficient to significantly inhibit the growth of Lactis^{Con} *in vitro* (Fig. 3B, C). In comparison, Lactis^{Lcn2} could better tolerate high concentrations of taurocholate and glycocholate up to 10 mM, and only displayed reduced growth in the presence of 100 mM of taurocholate and glycocholate *in vitro* (Fig. 3B, C). While most of the primary bile acids are reabsorbed by the small intestine, a fraction of these bile acids are metabolized by intestinal bacteria into secondary bile acids, such as deoxycholate and lithocholate [43] which could inhibit bacterial growth. Interestingly, we observed that Lactis^{Lcn2} could also better withstand the presence of lithocholate and deoxycholate at concentrations ranging from 0.001 to 0.1 mM *in vitro*, when compared with Lactis^{Con} (Fig. 3D, E).

In the context of experimental colitis, many probiotics and gut bacteria would be exposed to reactive oxygen species (ROS) generated by immune and non-immune cells in the inflamed gut. To assess whether Lcn2 expression in *L. lactis* exerts antioxidative effects, we next challenged Lactis^{Lcn2} and Lactis^{Con} with a range of oxidizing conditions. The viability of both Lactis^{Lcn2} and Lactis^{Con} decreased dose-dependently with increasing H_2O_2 concentrations, yet Lactis^{Lcn2} consistently survived better than Lactis^{Con} (Fig. 3F). When the assay was performed with 1.25 mM H_2O_2 under aerobic conditions (Fig. 3G), we observed that the viability of Lactis^{Con} decreased from 40% (without aeration) to 20% (with aeration). This outcome is expected, since the presence of O_2 in the aerated culture generates ROS, such as H_2O_2, OH^-, and O_2^-, which are unfavorable for *L. lactis*. However, 1.25 mM H_2O_2-treated Lactis^{Lcn2} was able to better withstand aeration with a slight decrease in viability from 65% to 55% (Fig. 3G). When the H_2O_2 concentration was increased to 2.5

mM with aeration, the viability of Lactis[Lcn2] decreased to 30%, whereas Lactis[Con] displayed only 10% viability (Fig. 3G). Next, we tested the extent to which Lactis[Lcn2] and Lactis[Con] can survive bacterial killing by neutrophil MPO, an oxidative enzyme which uses H_2O_2 and halide ions to generate hypochlorous acid [44]. As anticipated, Lactis[Lcn2] displayed a higher capacity to remain viable (80%) than Lactis[Con] (40%), even when exposed to MPO-H_2O_2 mediated antimicrobial activity (Fig. 3H).

Lcn2 expression allows *L. lactis* to colonize and survive better in the inflamed gut

To assess the survivability of Lactis[Lcn2] in the inflamed gut, we first induced colitis in 8 weeks old female WT mice (n=6) via administration of low dose of DSS (1.5%) in drinking water for 7 days. The induction of acute colitis was confirmed by the presence of occult blood and body weight loss. DSS treatment was withdrawn on day 7, and mice were maintained on regular drinking water. On the next day, the mice were orally administered with Lactis[Lcn2] or Lactis[Con] (1.0x10^{8} CFU/mouse), and fecal shedding of these bacteria was monitored. Interestingly, the fecal shedding of Lactis[Lcn2] was more robust and persisted up to 96 h post-inoculation when compared to Lactis[Con] (Fig. 4A). To verify the colonization capacity of Lactis[Lcn2] and Lactis[Con] in the inflamed gut, we next measured their bacterial load in the cecal content and colonic tissue. Our results showed that Lactis[Lcn2] can colonize in the colonic mucosa, in a more efficient manner than Lactis[Con] (Fig. 4B). Lactis[Lcn2] also colonized more robustly than Lactis[Con] in the ceca (Fig 4C). We next recovered the Lactis[Lcn2] from colitic mice and confirmed that it retained its Lcn2 expression (Fig. 4D). We next evaluated their tolerance to H_2O_2 exposure in order to assess whether Lactis[Lcn2] recovered from colitic mice retained their oxidant-resistant phenotype. Consistent with our previous findings, the recovered-Lactis[Lcn2] exhibited greater resistance to H_2O_2 *in vitro* than the recovered-Lactis[Con] (Fig. 4E).

Lcn2-expressing *L. lactis* accelerate mice recovery from DSS-induced acute colitis

Having established that Lactis[Lcn2] can survive and colonize the inflamed gut, we next investigated the therapeutic potential of Lactis[Lcn2] in attenuating intestinal inflammation in comparison to Lactis[Con]. First, we verified that the sole inoculation of Lactis[Lcn2] and Lactis[Con] into healthy mice did not result in diarrhea, colomegaly or splenomegaly (data not shown). Next, we performed DSS-induced colitis with 8 weeks old female WT (n=6), as described above. After confirming the onset of colitis on day 7, DSS was withdrawn, and mice were orally administered with Lactis[Lcn2] or Lactis[Con] (1.0x10^{8} CFU/mouse) therapeutically. Mice were monitored for body weight for another 4 days and then euthanized. We did not observe any significant difference in body weight gain/loss in Lactis[Lcn2] or Lactis[Con]-treated mice when compared to DSS-alone given mice (Fig. 5A). However, the treatment with Lactis[Lcn2], but not Lactis[Con], successfully mitigated the splenomegaly, colon shortening, and colonic MPO activity that are associated with DSS-induced acute colitis (Fig. 5B–F). Given that Lactis[Lcn2] was administered after DSS-induced colon shortening had already occurred, it is thus possible that Lactis[Lcn2] treatment may either prevent further shortening of the colon or promote the recovery, including restoration of colon length. In comparison to Lactis[Lcn2] treated mice, the mice receiving Lactis[Con] displayed only modest amelioration of colitis in all parameters analyzed. Histological (H&E) analysis indicated that the colitis in DSS+Lactis[Lcn2] treated mice was less severe,

when compared to DSS-alone and DSS+ Lactis[Con] treated mice (Fig. 5G,I). Similarly, the number of mature goblet cells containing large mucosal granules, as evident with Alcian blue staining, were markedly increased in DSS+ Lactis[Lcn2] treated mice compared with the DSS-alone and DSS+Lactis[Con] treated mice (Fig. 5H).

To further assess the effect of Lactis[Lcn2] on colitis, we evaluated a panel of systemic and colonic markers that correlate with the severity of inflammation. Colitic mice therapeutically treated with either Lactis[Lcn2] or Lactis[Con] displayed a significant decrease in levels of serum KC (Fig. 5J). The levels of SAA were also substantially reduced in Lactis[Lcn2] treated mice compared with Lactis[Con] treated mice (Fig. 5K). Furthermore, the colonic expression of inflammatory genes such as *KC*, tumor necrosis factor alpha (*TNFα*) and inducible nitric oxide synthase (*iNOS*) was reduced in both Lactis[Lcn2] and Lactis[Con]-treated mice, although their downregulation were slightly more pronounced in the Lactis[Lcn2] given mice (Fig. 5L, M & N).

Discussion

Intestinal inflammation is accompanied with systemic hypoferremia as an acute phase response to deplete circulating iron and prevent iron-induced oxidative stress. Such state of iron deficiency substantially impacts the composition of gut bacteria since iron is an essential micronutrient for almost all microorganisms, except *Borrelia* species and most lactic acid bacteria [45, 46]. During this iron-deprived state, the opportunistic pathogen *E. coli* produce Ent, a siderophore, to acquire the iron from the host and thereby possess survival advantage in the inflamed gut. On the flip side, the host relies on the innate immune protein Lcn2 to sequester Ent and curtail the overgrowth of *E. coli*. In comparison, *L. lactis* are largely unaffected by the antimicrobial activity of Lcn2 since *L. lactis* neither require iron nor express any siderophore[39, 40]. However, the inability of *L. lactis* to respond to iron status in the gut microenvironment would allow it to be rapidly out-competed by *E. coli* and other iron-responsive bacteria in the inflamed gut [47]. In this study, we have engineered *L. lactis* to express Lcn2 (Lactis[Lcn2]) as an innovative strategy to augment the fitness and efficacy of *L. lactis* in mitigating gut inflammation.

Given its small size (25 kDa) and the lack of post-translational modification requirement, Lcn2 presents a feasible candidate to be expressed in bacteria. We considered the food-grade *L. lactis* as the ideal vector strain since it is Gram-positive (endotoxin-free), non-pathogenic, non-invasive, and is a transient colonizer having probiotic properties. Most importantly, the lack of iron requirement and siderophore expression in *L. lactis* [39, 40] ensures that recombinant Lcn2 would be secreted in its active form that is free from iron and siderophore. We noted that Lactis[Lcn2] is capable of producing both the monomeric and dimeric forms of Lcn2, albeit the dimeric form was noticeably more abundant within the cell lysates. Although the functional differences between monomeric and dimeric forms of Lcn2 are yet to be clarified[37, 38], our *in vitro* assays nonetheless confirmed that Lactis[Lcn2]-derived Lcn2 is indeed bioactive in inhibiting the growth of *E. coli* and mitigating Ent bioactivity. Rather intriguingly, the expression of Lcn2 seemed to confer *L. lactis* with the ability to sequester iron from its microenvironment. Lcn2 alone, however, does not bind iron directly but requires siderophores as co-factors to form a 'Lcn2-siderophore-iron' ternary complex.

The accumulation of iron in *L. lactis* hence implies the involvement of an unknown siderophore, either generated by the bacterium or acquired from the media, which need to be further investigated for its role in facilitating iron chelation by Lcn2 in Lactis^{Lcn2}. Nevertheless, the ability of Lcn2 to augment iron-responses of *L. lactis* may be beneficial in allowing it to compete better with other iron-dependent bacteria in the gut [47].

Aside from its antimicrobial functions, Lcn2 overexpression has been demonstrated to enhance cellular resistance to oxidative stress and iron-induced toxicity in various mammalian cell lines[32–36, 48, 49]. As anticipated, the expression of Lcn2 substantially increased the capacity of Lactis^{Lcn2} to tolerate adverse conditions, including extreme pH, exposure to high concentrations of bile acids, oxidizing environments and MPO-mediated bacterial killing *in vitro*. The cytoprotective properties of Lcn2 are not well-understood, although several studies had associated its beneficial effects to its ability to modulate the labile iron pool and to exert anti-oxidative properties[32–36, 48, 49]. The changes in extracellular pH, for instance, could cause a fraction of lactic acid to become protonated, which enters and accumulates in the bacteria, thus triggering stress[41]. In this regard, we surmise that the growth advantage of Lactis^{Lcn2} over Lactis^{Con} at adverse pH could be possibly due to the mitigation of pH-mediated stress in the presence of Lcn2 acting as an antioxidant. We also presumed that similar antioxidant properties of Lcn2 may also increase the capacity of Lactis^{Lcn2} to tolerate the adverse oxidizing conditions tested in this study. Additionally, the survival of Lactis^{Lcn2}, in the large intestine, was more in the DSS given mice than Lactis^{Con}. More importantly, Lactis^{Lcn2} administration substantially reduced the severity of DSS-induced colitis in mice when compared to Lactis^{Con}, suggesting that the increased fitness of Lactis^{Lcn2} in colonizing the inflamed gut with a higher retention rate could, in part, contribute to the better amelioration of colitis. These findings provide evidence that successful colonization and fitness of recombinant probiotic bacteria in the inflamed gut is an important consideration when optimizing probiotics regimen.

Lcn2 primarily controls the gut bacterial overgrowth by limiting its iron uptake and also mediates facilitation of the hypoferremia during inflammation [12]. Additionally, Lcn2 serve as an indispensable factor for proper neutrophil migration, adhesion, and function[50]. In our recent study[16, 38] as well as others[17, 51], Lcn2 has been demonstrated to mediate an essential role in maintaining gut homeostasis, whereas its deficiency increases their susceptibility to colitis in mice. Considering that endogenous Lcn2 are significantly upregulated during IBD[2, 3], it is, therefore, unlikely that the delivery of additional Lcn2 via *L. lactis per se* could adequately account for the therapeutic properties of Lactis^{Lcn2}. Our findings suggested that the beneficial effects of Lactis^{Lcn2} could be potentially explained, at least in part, by the augmented survival or colonization ability of the probiotic *L. lactis* that express Lcn2. Nevertheless, we could not rule out the possibility that exogenous Lcn2 may also provide mucoprotective effects to the host via: (i) mitigating gut dysbiosis such as preventing the bloom of *Enterobacteriaceae* (e.g., *E. coli*)[4, 10], (ii) restricting luminal iron and the labile iron pool[12], thus attenuating iron-induced oxidative stress, (iii) serving as an epithelial survival factor [52] and promote wound healing[53, 54]. Further prolonged survival may also enhance the generation of *L. lactis* derived soluble, effector molecules, which can diminish gut inflammation.

Most probiotics tested in clinical trials failed to improve human IBD[55], despite their efficacy in mitigating the gut inflammation in experimental animal models. The lack of success in the clinical setting may be explained by the inherent deficiency of these probiotics to survive against the host inflammatory responses. Such notion coincides with the consideration that many candidate probiotics are selected from bacteria that transiently colonizes the gut, and thus may be rapidly depleted in the inflamed gut. In the present study, we demonstrated that Lcn2 expression could be engineered into *L. lactis* to enhance its survivability, colonization, and capacity to tolerate the harsh environments in the inflamed gut. Overall, our findings highlight the potential for Lcn2 expression or co-expression alongside other therapeutic molecules as an innovative strategy to further stabilize probiotic bacteria that are currently studied to mitigate intestinal inflammation and allow for better management of experimental colitis.

Acknowledgments

Abbreviations

APP	acute-phase protein
DSS	Dextran sodium sulfate
Ent	Enterobactin
IBD	Inflammatory bowel diseases
L. lactis	Lactococcus lactis
Lcn2	Lipocalin 2
Lactis^{Lcn2}	Lipocalin 2 expressing *L. lactis*
Lactis^{Con}	*L. lactis* transformed with empty plasmid
MPO	Myeloperoxidase
ROS	Reactive oxygen species

References

1. Vermeire S, Van Assche G, Rutgeerts P. Laboratory markers in IBD: useful, magic, or unnecessary toys? Gut. 2006; 55:426–431. [PubMed: 16474109]

2. Chakraborty S, Kaur S, Guha S, et al. The multifaceted roles of neutrophil gelatinase associated lipocalin (NGAL) in inflammation and cancer. Biochim Biophys Acta. 2012; 1826:129–169. [PubMed: 22513004]

3. Chassaing B, Srinivasan G, Delgado MA, et al. Fecal lipocalin 2, a sensitive and broadly dynamic non-invasive biomarker for intestinal inflammation. PLoS One. 2012; 7:e44328. [PubMed: 22957064]

4. Flo TH, Smith KD, Sato S, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature. 2004; 432:917–921. [PubMed: 15531878]

26. Singh V, Yeoh BS, Xiao X, et al. Interplay between enterobactin, myeloperoxidase and lipocalin 2 regulates E. coli survival in the inflamed gut. Nat Commun. 2015; 6:7113. [PubMed: 25964185]

27. Lee SK, Keasling JD. A propionate-inducible expression system for enteric bacteria. Appl Environ Microbiol. 2005; 71:6856–6862. [PubMed: 16269719]

28. Chassaing B, Aitken JD, Malleshappa M, et al. Dextran sulfate sodium (DSS)-induced colitis in mice. Curr Protoc Immunol. 2014; 104(Unit 15):25. [PubMed: 24510619]

29. Singh V, Yeoh BS, Carvalho F, et al. Proneness of TLR5 deficient mice to develop colitis is microbiota dependent. Gut Microbes. 2015; 6:279–283. [PubMed: 26067589]

30. Cooper HS, Murthy SN, Shah RS, et al. Clinicopathologic study of dextran sulfate sodium experimental murine colitis. Lab Invest. 1993; 69:238–249. [PubMed: 8350599]

31. Arroyo A, Modriansky M, Serinkan FB, et al. NADPH oxidase-dependent oxidation and externalization of phosphatidylserine during apoptosis in Me2SO-differentiated HL-60 cells. Role in phagocytic clearance. J Biol Chem. 2002; 277:49965–49975. [PubMed: 12376550]

32. Cao Z, Yu W, Li W, et al. Oxidative Damage and Mitochondrial Injuries Are Induced by Various Irrigation Pressures in Rabbit Models of Mild and Severe Hydronephrosis. PLoS One. 2015; 10:e0127143. [PubMed: 26090815]

33. Ebrahimi-Kalan A, Soleimani Rad J, Kafami L, et al. MS14 down-regulates lipocalin2 expression in spinal cord tissue in an animal model of multiple sclerosis in female C57BL/6. Iran Biomed J. 2014; 18:196–202. [PubMed: 25326017]

34. Halabian R, Tehrani HA, Jahanian-Najafabadi A, et al. Lipocalin-2-mediated upregulation of various antioxidants and growth factors protects bone marrow-derived mesenchymal stem cells against unfavorable microenvironments. Cell Stress Chaperones. 2013; 18:785–800. [PubMed: 23620204]

35. Miyamoto T, Kashima H, Yamada Y, et al. Lipocalin 2 Enhances Migration and Resistance against Cisplatin in Endometrial Carcinoma Cells. PLoS One. 2016; 11:e0155220. [PubMed: 27168162]

36. Macedo-Marquez A, Vazquez-Acevedo M, Ongay-Larios L, et al. Overexpression of a monomeric form of the bovine odorant-binding protein protects Escherichia coli from chemical-induced oxidative stress. Free Radic Res. 2014; 48:814–822. [PubMed: 24697800]

37. Martensson J, Xu S, Bell M, et al. Immunoassays distinguishing between HNL/NGAL released in urine from kidney epithelial cells and neutrophils. Clin Chim Acta. 2012; 413:1661–1667. [PubMed: 22609864]

38. Saha P, Singh V, Xiao X, et al. Data on importance of hematopoietic cell derived Lipocalin 2 against gut inflammation. Data Brief. 2016; 8:812–816. [PubMed: 27500193]

39. Pandey A, Bringel F, Meyer JM. Iron Requirement and Search for Siderophores in Lactic-Acid Bacteria. Applied Microbiology and Biotechnology. 1994; 40:735–739.

40. Bruyneel B, Vandewoestyne M, Verstraete W. Lactic-Acid Bacteria - Microorganisms Able to Grow in the Absence of Available Iron and Copper. Biotechnology Letters. 1989; 11:401–406.

41. Madsen SM, Arnau J, Vrang A, et al. Molecular characterization of the pH-inducible and growth phase-dependent promoter P170 of Lactococcus lactis. Mol Microbiol. 1999; 32:75–87. [PubMed: 10216861]

42. Yokota A, Veenstra M, Kurdi P, et al. Cholate resistance in Lactococcus lactis is mediated by an ATP-dependent multispecific organic anion transporter. J Bacteriol. 2000; 182:5196–5201. [PubMed: 10960105]

43. Brinkmann V, Reichard U, Goosmann C, et al. Neutrophil extracellular traps kill bacteria. Science. 2004; 303:1532–1535. [PubMed: 15001782]

44. Klebanoff SJ, Kettle AJ, Rosen H, et al. Myeloperoxidase: a front-line defender against phagocytosed microorganisms. J Leukoc Biol. 2013; 93:185–198. [PubMed: 23066164]

45. Weinberg ED. The Lactobacillus anomaly: total iron abstinence. Perspect Biol Med. 1997; 40:578–583. [PubMed: 9269745]

46. Posey JE, Gherardini FC. Lack of a role for iron in the Lyme disease pathogen. Science. 2000; 288:1651–1653. [PubMed: 10834845]

47. Bailey JR, Probert CS, Cogan TA. Identification and characterisation of an iron-responsive candidate probiotic. PLoS One. 2011; 6:e26507. [PubMed: 22039501]

48. Bahmani B, Roudkenar MH, Halabian R, et al. Lipocalin 2 decreases senescence of bone marrow-derived mesenchymal stem cells under sub-lethal doses of oxidative stress. Cell Stress Chaperones. 2014; 19:685–693. [PubMed: 24452457]

49. Hu L, Hittelman W, Lu T, et al. NGAL decreases E-cadherin-mediated cell-cell adhesion and increases cell motility and invasion through Rac1 in colon carcinoma cells. Lab Invest. 2009; 89:531–548. [PubMed: 19308044]

50. Schroll A, Eller K, Feistritzer C, et al. Lipocalin-2 ameliorates granulocyte functionality. Eur J Immunol. 2012; 42:3346–3357. [PubMed: 22965758]

51. Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of siderophores. Anal Biochem. 1987; 160:47–56. [PubMed: 2952030]

52. Tong Z, Wu X, Ovcharenko D, et al. Neutrophil gelatinase-associated lipocalin as a survival factor. Biochem J. 2005; 391:441–448. [PubMed: 16060857]

53. Miao Q, Ku AT, Nishino Y, et al. Tcf3 promotes cell migration and wound repair through regulation of lipocalin 2. Nat Commun. 2014; 5:4088. [PubMed: 24909826]

54. Playford RJ, Belo A, Poulsom R, et al. Effects of mouse and human lipocalin homologues 24p3/lcn2 and neutrophil gelatinase-associated lipocalin on gastrointestinal mucosal integrity and repair. Gastroenterology. 2006; 131:809–817. [PubMed: 16952550]

55. Jonkers D, Penders J, Masclee A, et al. Probiotics in the management of inflammatory bowel disease: a systematic review of intervention studies in adult patients. Drugs. 2012; 72:803–823. [PubMed: 22512365]
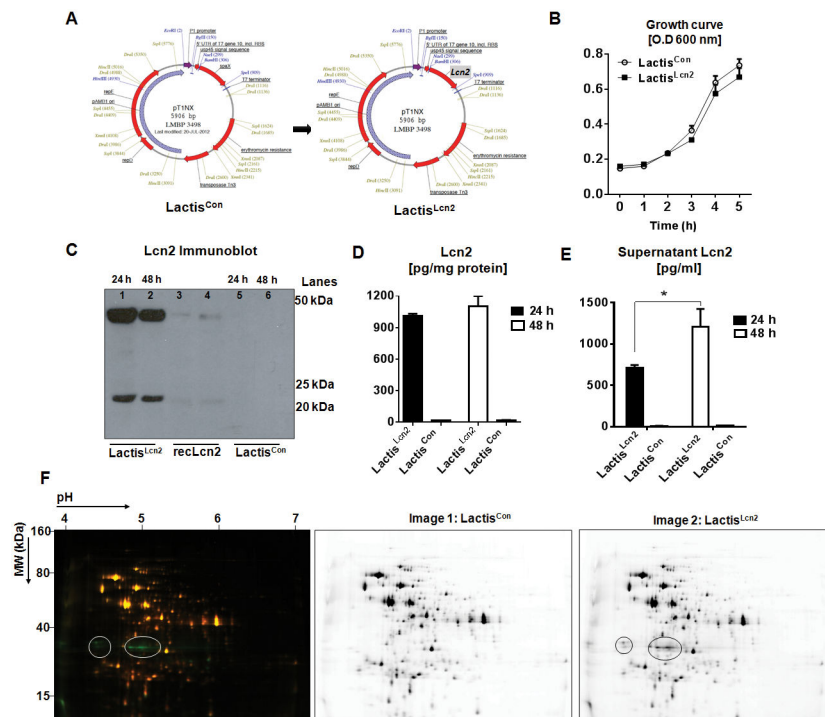
Figure 1. Schematic representation and characterization of Lcn2 expressing *L. lactis*
(A) Murine Lcn2 gene was cloned into pT1NX plasmid at BamH1/Spe1 sites downstream of P1 promoter and USP45 signal sequence to allow for constitutive expression and secretion, respectively. **(B)** Line graphs represent the growth curve for the recombinant Lactis[Lcn2] and Lactis[Con] cultured in M17 media supplemented with erythromycin. Lactis[Lcn2] and Lactis[Con] were cultured for 24 and 48 h, and Lcn2 levels were assayed in the cell lysates by **(C)** immunoblotting and by **(D)** ELISA, and **(E)** culture supernatant by ELISA. Mouse recombinant (rec)-Lcn2 was used as a positive control for the Lcn2 immunoblot. **(F)** Composite overlay representative 2D-DIGE image (left) and individual images (right) showing protein accumulation in Lactis[Con] (Cy5, red fluorescence) and Lactis[Lcn2] (Cy3, green fluorescence) lysates. Green spots represent proteins up-regulated in Lactis[Lcn2] and yellow spots represent proteins that are not deregulated. Circles indicate the protein bands predicted to be Lcn2. All experiments were performed in triplicates and are representative of three independent experiments. Results are expressed as mean ± SEM; *p<0.05.
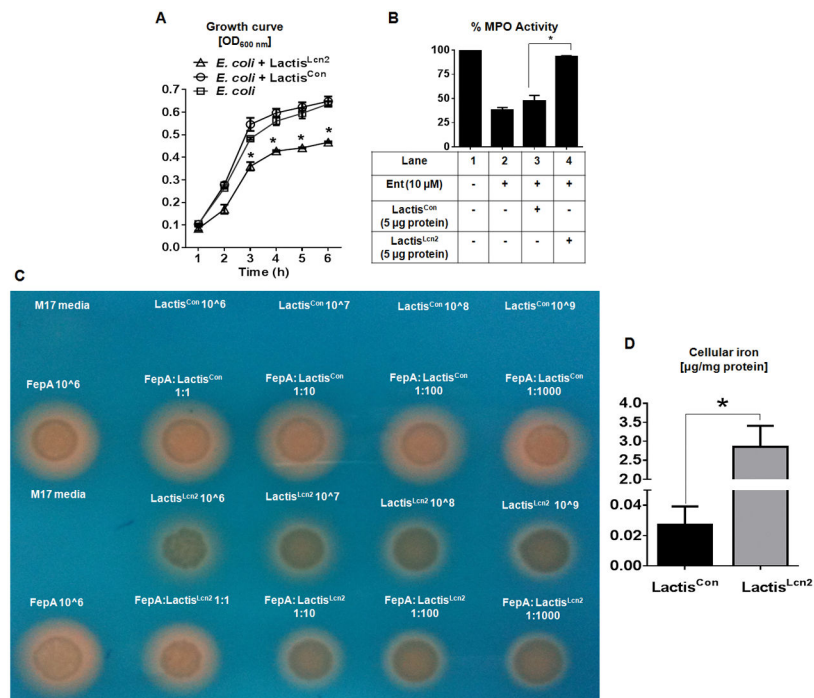
Figure 2. Lcn2 produced by Lactis[Lcn2] is bioactive

(A) Line graph represents the growth curve for *E. coli* cultured in the presence of cell lysates (5 μg of protein) from Lactis[Lcn2], Lactis[Con] or vehicle control. Myeloperoxidase (MPO, 100 mU) were pre-incubated with enterobactin (Ent, 10 μM) and cell lysates (5 μg of protein) from Lactis[Lcn2] or Lactis[Con] for 5 min before the assay was initiated. **(B)** Bar graph represents % MPO activity. **(C)** Orange halo formation indicates siderophore production by *fepA E. coli* mutant on CAS plate (cultured overnight, 37°C) in the presence of Lactis[Lcn2], Lactis[Con] or vehicle control at the indicated CFU concentration/ratio. **(D)** The cellular iron levels were measured in the cell lysates from Lactis[Lcn2] and Lactis[Con]. All experiments were performed in triplicates and are representative of three independent experiments. Results are expressed as mean ± SEM. *$p < 0.05$.
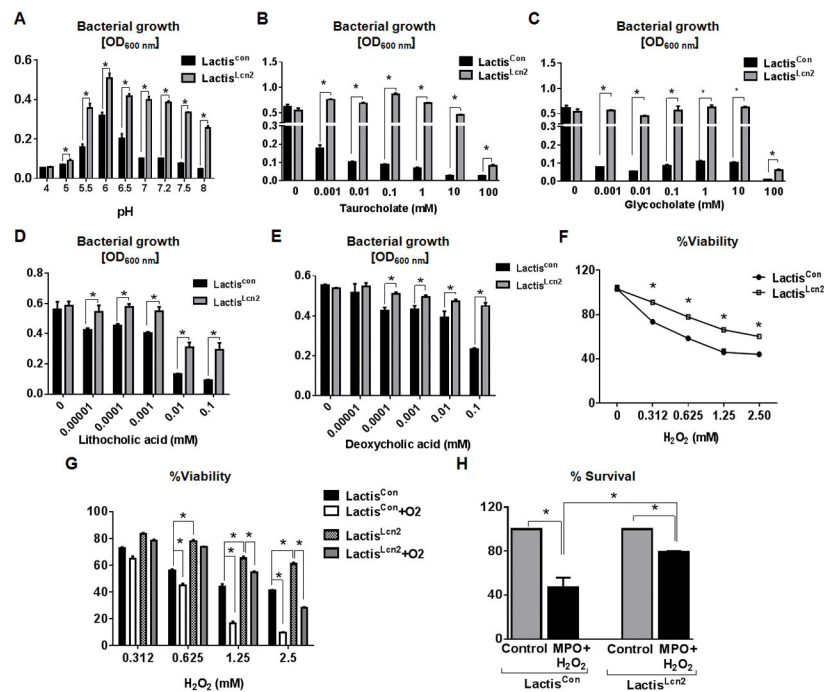
Figure 3. Lcn2 expressing *L. lactis* (Lactis^Lcn2) exhibits augmented resistance to various stressors

Equal amount of Lactis^Lcn2 or Lactis^Con (1 x 10^6 CFU) were cultured at the indicated **(A)** pH, or in the presence of **(B)** taurocholate, **(C)** glycocholate, **(D)** lithocholic acid and **(E)** deoxycholic acid at the indicated concentration. Lactis^Lcn2 or Lactis^Con were subjected to the indicated concentration of **(F)** H_2O_2, **(G)** H_2O_2 with aeration, and **(H)** myeloperoxidase (MPO)-mediated bacterial killing. All experiments were performed in triplicates and are representative of three independent experiments. Results are expressed as mean ± SEM. *$p < 0.05$.
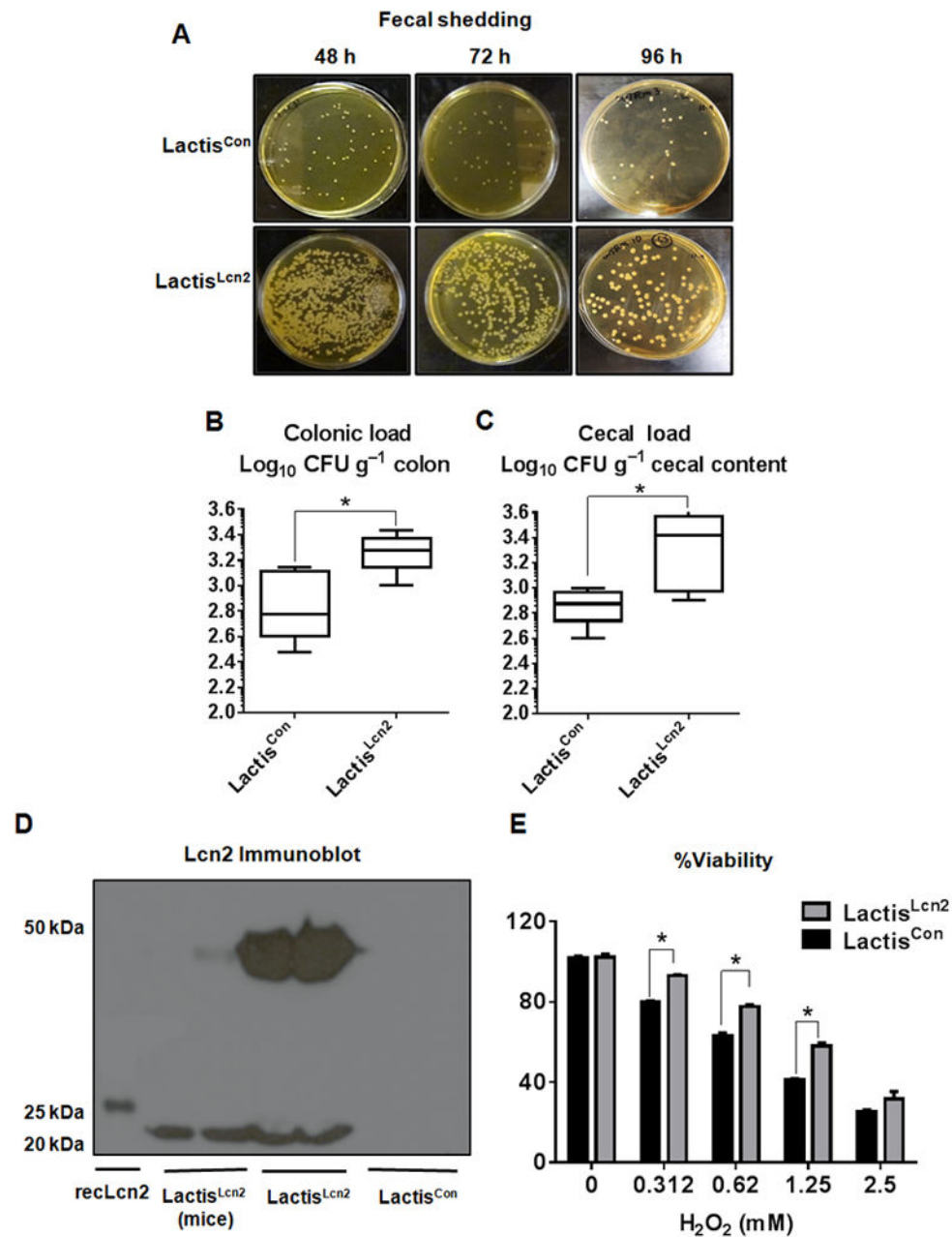
Figure 4. Lcn2 expression provides a survival advantage to Lactis^{Lcn2} in the inflamed gut

DSS-induced colitic female WT mice (n=6) were orally administered with Lactis^{Lcn2} or Lactis^{Con}. **(A)** Fecal shedding collected at 48, 72 and 96 h post-inoculation were plated on selective M17 plates containing erythromycin. Bacterial load in the **(B)** colonic mucosa and **(C)** cecal content were measured on selective M17 plates. **(D)** Lcn2 expression in the cell lysates of Lactis^{Lcn2} recovered from colitic mice feces (96 h, DSS+Lactis^{Lcn2}) was assayed by immunoblotting. Mouse recombinant (rec)-Lcn2 and the parent stock of Lactis^{Lcn2} were used as positive controls for the Lcn2 immunoblot. **(E)** Lactis^{Lcn2} or Lactis^{Con} recovered from colitic mice were subjected to the indicated concentration of H_2O_2 *in vitro*. All

experiments were performed in triplicates and are representative of three independent experiments. Results are expressed as mean ± SEM. *p < 0.05.
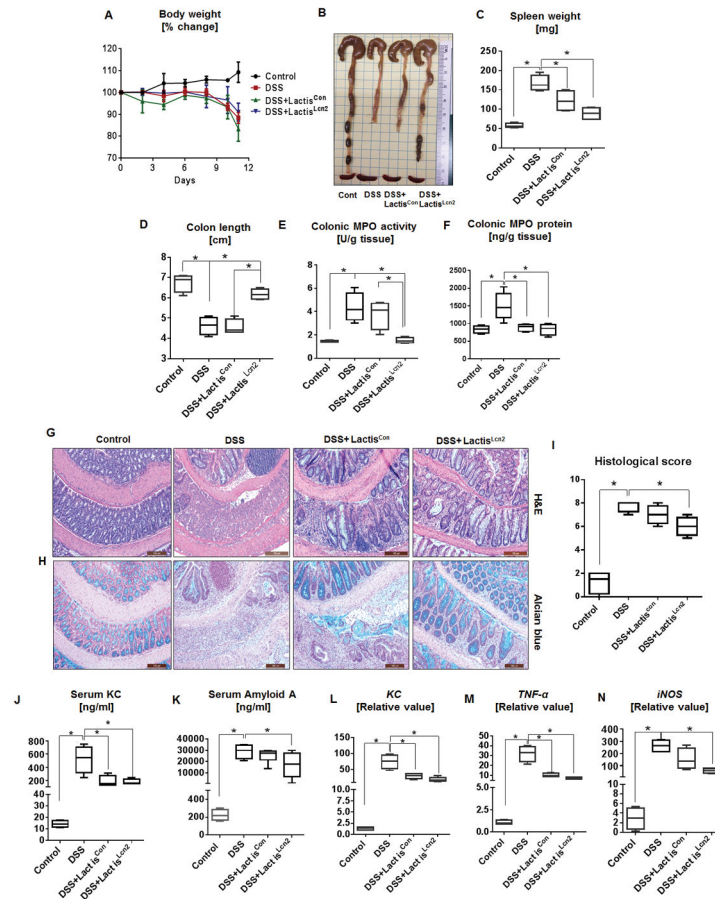
Figure 5. Lcn2 expressing *L. lactis* protects against DSS-induced mucosal injury

Eight weeks old female BL6 WT mice (n=6) were given 1.5% DSS in drinking water for 7 days. After cessation of DSS on day 7, mice were maintained on regular water. On day 8 onwards, mice were orally administered with Lactis[Lcn2] or Lactis[Con] (1×10^8 CFU/mouse) once daily. The following parameters were analyzed **(A)** body weight, **(B)** gross colon, **(C)** spleen weight, **(D)** colon length, **(E)** colonic MPO activity and **(F)** colonic MPO protein level. Histology images of **(G)** H&E-stained and **(H)** Alcian blue-stained colons. **(I)** Histological scores of H&E-stained colons. *In vitro* assays were performed in triplicates and are representative of two independent mouse experiments. The perceptive markers of intestinal inflammation: **(J)** serum KC, and **(K)** SAA were measured by ELISA. The qRT-PCR analysis was used to measure the mRNA expression of colonic **(L)** *KC*, **(M)** *TNF-α* and **(N)** *iNOS*. Values are represented as fold change normalized to *36B4* housekeeping gene and compared to the control group. *In vitro* assays were performed in triplicates and are representative of two independent mouse experiments. Results are expressed as mean ± SEM. *p<0.05.