Published in final edited form as:
Cell Host Microbe. 2013 July 17; 14(1): 26–37. doi:10.1016/j.chom.2013.06.007.

Probiotic Bacteria Reduce *Salmonella* Typhimurium Intestinal Colonization by Competing for Iron

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Summary

Host inflammation alters the availability of nutrients such as iron to limit microbial growth. However, *Salmonella enterica* serovar Typhimurium thrives in the inflamed gut by scavenging for iron with siderophores. By administering *Escherichia coli* strain Nissle 1917, which assimilates iron by similar mechanisms, we show that this non-pathogenic bacterium can outcompete and reduce *S.* Typhimurium colonization in mouse models of acute colitis and chronic persistent infection. This probiotic activity depends on *E. coli* Nissle iron acquisition as mutants deficient in iron uptake colonize the intestine but do not reduce *S.* Typhimurium colonization. Additionally, the ability of *E. coli* Nissle to overcome iron restriction by the host protein lipocalin-2, which counteracts some siderophores, is essential as *S.* Typhimurium is unaffected by *E. coli* Nissle in lipocalin-2-deficient mice. Thus, iron availability impacts *S.* Typhimurium growth and *E. coli* Nissle reduces *S.* Typhimurium intestinal colonization by competing for this limiting nutrient.

Introduction

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is one of the leading causes of acute gastroenteritis characterized by inflammatory diarrhea. While the normal intestine is largely inhabited by commensal microbes, which largely include *Bacteroides* and *Firmicutes*, inflammation enhances the colonization of *S.* Typhimurium and other *Enterobacteriaceae* (Barman et al., 2008; Lawley et al., 2008; Lupp et al., 2007; Stecher et al., 2007). Recent studies have shown that *S.* Typhimurium thrives in the inflamed gut because it can utilize unique carbon and energy sources (Thiennimitr et al., 2011; Winter et

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Supplemental information

Supplemental information includes five figures, three tables, Supplemental Experimental Procedures, and Supplemental References.

al., 2010) and is resistant to antimicrobial proteins that are secreted by the host as part of the nutritional immune response (Liu et al., 2012; Raffatellu et al., 2009). *S.* Typhimurium employs specialized transporters to acquire essential micronutrient metals (Liu et al., 2012; Raffatellu et al., 2009), one of the most important being iron (Crouch et al., 2008; Raffatellu et al., 2009). Levels of free iron are extremely low in the host environment due to sequestration by host proteins including heme, ferritin, transferrin and lactoferrin (Andrews and Schmidt, 2007). Additional mechanisms are employed by the host to further limit iron availability during inflammation (Weinberg, 1984), including secretion of the hormone hepcidin, which prevents the gut from absorbing iron from the bloodstream by inhibiting the iron transporter ferroportin-1 (Ganz, 2003).

When starved for iron, bacteria synthesize and export small-molecule high-affinity iron chelators termed siderophores. Enterochelin is a catecholate-type siderophore secreted by all *Enterobacteriaceae*, including *Salmonella* and commensal *E. coli* (Raymond et al., 2003), which is sufficient to overcome the host's iron limitation in a normal (non-inflamed) environment. However, during inflammatory responses, the host secretes lipocalin-2, an antimicrobial peptide that sequesters ferric enterochelin, thereby limiting the growth of strains like commensal *E. coli* that rely solely upon enterochelin for siderophore-based iron acquisition (Berger et al., 2006; Flo et al., 2004). Some pathogens evade this response by synthesizing additional siderophores that are not sequestered by lipocalin-2 (Fischbach et al., 2006a). For instance, *Salmonella* can synthesize and secrete salmochelin (Muller et al., 2009), a C-glucosylated derivative of enterochelin that is too large to fit into the enterochelin-binding pocket of lipocalin-2 (Fischbach et al., 2006b; Hantke et al., 2003).

Probiotics are commensal microorganisms that are believed to exert beneficial effects on the host. *Escherichia coli* Nissle 1917 (*E. coli* Nissle, serotype O6:K5:H1) is a probiotic strain that was originally isolated from a soldier who appeared resistant to an outbreak of diarrhea (Nissle, 1959). *E. coli* Nissle has been shown to establish persistent colonization of the intestine and has been used to treat or prevent a variety of intestinal disorders (Cukrowska et al., 2002; Kruis et al., 2004; Lodinova-Zadnikova and Sonnenborn, 1997; Mollenbrink and Bruckschen, 1994; Nissle, 1959), including acute enteritis (Henker et al., 2007), but the mechanistic basis for its protective actions is unknown. As salmochelin-mediated iron acquisition during inflammation enhances *S.* Typhimurium colonization (Raffatellu et al., 2009), we hypothesized that *E. coli* Nissle might protect the host by utilizing similar mechanisms to compete with *S.* Typhimurium for essential micronutrients.

A snapshot analysis of the *E. coli* Nissle genome revealed that it shares many fitness properties found in uropathogenic *E. coli* (UPEC) strains of the same serotype (Grozdanov et al., 2004). Intriguingly, the *E. coli* Nissle genome appears to encode for as many iron uptake systems as UPEC (Garcia et al., 2011), an armament that notably includes salmochelin, the hydroxamate-type siderophore aerobactin, the mixed-type siderophore yersiniabactin and the hemin uptake transporter ChuA. As redundancy in iron uptake promotes the growth of UPEC in the bladder and the kidney (Garcia et al., 2011), we reasoned that it may also contribute to *E. coli* Nissle colonization of the inflamed gut. To test the hypothesis that iron uptake mechanisms are important for *E. coli* Nissle probiotic activity, we set out to examine the effect of administering wild-type *E. coli* Nissle or mutant derivatives deficient in iron uptake on the course of *S.* Typhimurium infection.

Results

Probiotic *E. coli* Nissle 1917 reduces *S.* Typhimurium fecal shedding

Our prior studies indicated that *S.* Typhimurium must overcome iron limitation to successfully infect the host (Crouch et al., 2008; Raffatellu et al., 2009). To confirm that

iron limitation occurs during *S*. Typhimurium infection, we first measured the concentration of iron in the feces of mice four days after infection in an *S*. Typhimurium colitis model by inductively coupled plasma mass spectrometry (ICP-MS). We found that the concentration of fecal iron in the absence of infection was approximately 950 mg/kg (Fig. 1A). In contrast, the concentration of fecal iron was significantly reduced on average to 300 mg/kg in mice infected with *S*. Typhimurium (Fig. 1A), confirming that infection results in limitation of this metal in the colonic environment. To gain insight on the mechanism behind the lower levels of intestinal iron, we determined the expression of hepcidin and ferroportin-1 (Fig S1). While hepcidin transcripts were highly abundant in the liver of both mock-infected and *S*. Typhimurium-infected mice, the expression of hepcidin was not upregulated during *S*. Typhimurium infection (Fig S1). Nonetheless, we observed a modest but significant downregulation of ferroportin-1 in the liver and in the intestine of mice infected with *S*. Typhimurium, which should have resulted in higher levels of intestinal iron due to its reduced intestinal absorption. However, mice infected with *S*. Typhimurium also showed a significant weight loss and reduced food uptake (Fig S1 and data not shown), which may contribute to the lower concentration of iron in the intestine of infected mice (Fig 1A).

Once we established that intestinal iron is indeed limited during *S*. Typhimurium infection, we next assessed the effects of *E. coli* Nissle administration on *S*. Typhimurium infection. First, we utilized 129X1/SvJ mice, which develop chronic *Salmonella* colitis with persistent infection (Lawley et al., 2008). To ensure that all mice became highly colonized, streptomycin was administered prior to infection as previously described (Barthel et al., 2003; Lawley et al., 2008). *S*. Typhimurium-infected mice were then followed for three weeks post-infection and exhibited consistent high levels of fecal shedding of *Salmonella* (Fig. 1B) as well as high levels of inflammation (Fig. 2). Remarkably, a single therapeutic dose of wild-type *E. coli* Nissle administered three days after inoculation with *S*. Typhimurium was able to establish persistent colonization (Fig. 1C) and significantly reduce *S*. Typhimurium colonization by more than 2 logs for the duration of the study (Fig. 1C, 1E, **and** S1). To determine whether the beneficial effect of *E. coli* Nissle is dependent upon its ability to acquire iron, a mutation was constructed in the *tonB* gene of *E. coli* Nissle.

TonB provides the energy necessary for active transport of iron-laden siderophores and heme (Braun and Hantke, 2011). As expected, *in vitro* growth of *E. coli* Nissle *tonB* was equivalent to wild-type in an iron rich media (Fig. 3A **and** S2) or when *tonB* was complemented *in trans* (Fig. S2), but not in media when iron was limited by the addition of serum (Fig. 3C) or of by iron chelation with 2,2′-dipyridyl (Fig. 3E). Strikingly, *E. coli* Nissle *tonB* was still able to establish persistent colonization when administered to 129X1/SvJ mice three days after *S.* Typhimurium infection but was unable to reduce the *S.* Typhimurium burden in the feces (Fig. 1D, 1E, S1). Moreover, only wild-type *E. coli* Nissle was able to ameliorate the chronic inflammation observed in these S. Typhimurium-challenged mice at 22 days post-infection (Fig. 2). Consistent with this observation, transcript levels of *Lcn2*, which encodes for lipocalin-2, and levels of the pro-inflammatory cytokines *Tnf-α* and Ifn-γ were reduced in the cecum by wild-type *E. coli* Nissle but not by the *tonB* mutant (Fig. 2D). Therefore, our results suggested that iron acquisition is important for the probiotic activity of *E. coli* Nissle and for its ability to compete with *S*. Typhimurium in the inflamed gut during chronic infection.

Growth of *E. coli* Nissle strains in iron-rich and iron-limited media

As *tonB* mutations impede multiple iron acquisition mechanisms, a mutant strain was constructed which lacks four separate *tonB*-dependent iron transport systems that have been shown to contribute to iron acquisition during UPEC urinary tract infection (Garcia et al., 2011). The resulting strain lacks the salmochelin receptor IroN, the aerobactin receptor IutA,

the yersiniabactin receptor FuyA and the heme receptor ChuA (*E. coli* Nissle *iroN fyuA iutA chuA*) and it was termed *E. coli* Nissle Δ4. Notably, in contrast to the *tonB* mutant, *E. coli* Nissle Δ4 may acquire iron via ferric enterochelin through the FepA receptor. However, blockade of ferric enterochelin via lipocalin-2 should result in growth inhibition. Therefore, we determined the growth of the *E. coli* Nissle Δ4 in rich media supplemented with serum and/or the enterochelin scavenger lipocalin-2, as we previously described (Raffatellu et al., 2009). As expected, the *E. coli* Nissle Δ4 grew as well as wild-type in iron rich media (Fig. 3B **and** S2) and – in contrast to the *E. coli* Nissle *tonB* (Fig. 3C) - in rich media supplemented with serum (Fig. 3D), consistent with effective iron acquisition via ferric enterochelin. However, this strain exhibited a significant growth impairment in medium supplemented with the iron scavenger lipocalin-2 (Fig. 3F). Furthermore, the growth inhibition by lipocalin-2 on the *E. coli* Nissle Δ4 mutant was no longer observed if ferric iron was added to the media in the form of iron citrate (Fig. S2) (Pressler et al., 1988). Promisingly, the growth of wild-type *E. coli* Nissle was not impaired by supplementation with lipocalin-2 (Fig. 3, S2). Taken together, these results suggested that lipocalin-2 resistance mediated by non-enterochelin high-affinity iron uptake mechanisms might allow *E. coli* Nissle to grow in the inflamed gut and reduce *S.* Typhimurium colonization.

E. coli Nissle 1917 requires iron uptake systems to reduce *S.* Typhimurium intestinal colonization

To further assess whether high affinity iron transporters may contribute to the probiotic effect of *E. coli* Nissle, we administered wild-type *E. coli* Nissle or isogenic mutant strains deficient in iron uptake to mice infected with *S.* Typhimurium in a model of acute colitis. Specifically, C57BL/6 mice were administered streptomycin one day prior to infection as previously described(Barthel et al., 2003; Raffatellu et al., 2009). Mice were then infected with *S.* Typhimurium alone or co-administered with an equal dose of *E. coli* Nissle wild-type or mutants (Figure 4 **and** S3). Colonization in the colon content was then determined at 48, 72 and 96 hours after infection. In this model of acute colitis, the administration of *E. coli* Nissle significantly reduced intestinal colonization with *S.* Typhimurium at every time point (Fig. 4A, 4B, **and** S3). In contrast, neither the *E. coli* Nissle *tonB* nor the Δ4 mutants were able to reduce intestinal *S.* Typhimurium colonization (Fig. 4A, 4B, **and** S3) despite successfully establishing intestinal colonization (Fig. 4A, 4B, **and** S3). Of note, while *E. coli* Nissle wild-type outgrew *S.* Typhimurium, both *E. coli* Nissle *tonB* and the Δ4 mutant were outcompeted by *S.* Typhimurium at 96 hours after infection (see also Fig. 7). Moreover, complementation of *E. coli* Nissle *tonB in trans* partly rescued this strain's capability to reduce *S.* Typhimurium colonization (Fig. 4C, 4D, **and** S3).

To gain further insight into the competition for iron between *S.* Typhimurium and *E. coli* Nissle in the inflamed gut, we tested whether *E. coli* Nissle would affect the colonization of an *S.* Typhimurium strain lacking the IroN receptor (*iroN* mutant), which cannot acquire iron via salmochelin ((Raffatellu et al., 2009) and Fig. S2). We have previously shown that an *S.* Typhimurium *iroN* mutant has a colonization defect in the inflamed gut that is dependent on the expression of lipocalin-2 (Raffatellu et al., 2009). Consistent with our earlier study, we found that an *S.* Typhimurium *iroN* mutant showed a defect in colonization when compared to *S.* Typhimurium wild-type (Fig. 4C, 4D, **and** S3). Consistent with our present study, administration of *E. coli* Nissle did not further reduce colonization of the *S.* Typhimurium *iroN* mutant, supporting the notion that competition for iron is an essential trait of its probiotic activity (Fig. 4C, 4D, **and** S3). Of note, at 96 hours post-infection, the colonization of the *S.* Typhimurium *iroN* mutant further decreased because of the higher levels of lipocalin-2 (Fig. 4D **and** S3C). In contrast, the downregulation of lipocalin-2 caused by the administration of *E. coli* Nissle partly rescued the *iroN* mutant, which however is still significantly lower than wild-type *S.* Typhimurium at this time point; this is

consistent with our previously published results showing that the *iroN* mutant is rescued in *Lcn2*$^{-/-}$ mice (Raffatellu et al., 2009). Taken together, these results demonstrate that iron uptake is crucial for the beneficial effect of *E. coli* Nissle in reducing the colonization of *S.* Typhimurium.

As *E. coli* Nissle was able to diminish the intestinal burden of *S.* Typhimurium, we next analyzed whether the probiotic strain also ameliorated the intestinal inflammation caused by *S.* Typhimurium in the acute colitis model (Fig. 5, S4). Administration of *E. coli* Nissle alone did not cause intestinal inflammation (Fig. 5, S4). Intriguingly, the addition of wild-type *E. coli* Nissle, *E. coli* Nissle *tonB* or *E. coli* Nissle Δ4 each resulted in similar reductions of intestinal inflammation in *S.* Typhimurium-infected C57BL/6 mice (Fig. 5A, B, D, E, S4). In accordance with the reduction in inflammation, decreased transcript levels of *Lcn2*, of the neutrophil chemoattractant *Cxcl-1*, and of the pro-inflammatory cytokine *Il-17a* were also observed (Fig. 5C, F). To determine whether *E. coli* Nissle has direct anti-inflammatory effects, mice treated with dextran sodium sulfate (DSS) in the drinking water as a means to induce colitis in the absence of a bacterial infection were administered a single dose of *E. coli* Nissle (Wirtz et al., 2007). Similar to *E. coli* Nissle administration during *S.* Typhimurium infection, treatment with *E. coli* Nissle reduced the expression of pro-inflammatory cytokines in DSS-treated mice (Fig S4). Based on these results, it appears that *E. coli* Nissle exerts beneficial effects on cecal inflammation during acute *Salmonella* colitis that are independent of its antagonism of *S.* Typhimurium colonization.

The host inflammatory response limits iron availability to invading microbes in what is known as nutritional immunity (Cassat and Skaar, 2013). One arm of the nutritional immune response during infection is the upregulation of lipocalin-2, which, as described earlier, sequesters iron-bound enterochelin. We previously demonstrated that the ability to acquire iron through salmochelin provides a colonization advantage to *S.* Typhimurium wild-type in competition with an *iroN* mutant in mice that express lipocalin-2, but not in *Lcn2*$^{-/-}$ mice, likely because enterochelin alone is sufficient to overcome other mechanisms of ferric iron restriction in the host (Raffatellu et al., 2009). Consistent with this notion, intestinal *S.* Typhimurium colonization in wild-type mice was not affected by administration of a non-probiotic commensal *E. coli* strain (Fig. 6A) that relies on enterochelin for ferric iron acquisition and is thus susceptible to lipocalin-2 (Berger et al., 2006; Flo et al., 2004). We therefore hypothesized that *E. coli* Nissle would lose its competitive advantage over *S.* Typhimurium in the absence of lipocalin-2. As predicted, intestinal *S.* Typhimurium colonization in *Lcn2*$^{-/-}$ mice was not decreased by the administration of *E. coli* Nissle, despite successful colonization of the mouse intestine by this probiotic strain (Fig. 6B). As we previously showed, wild-type and *Lcn2*$^{-/-}$ mice infected with *S.* Typhimurium did not show significant differences in *S.* Typhimurium colonization (Fig. 4, S3, and 6B) or inflammation (Fig. S4 and S5). Furthermore, the expression of pro-inflammatory cytokines was similar between wild-type mice and *Lcn2*$^{-/-}$ mice, with the exception of *Cxcl-1,* which was reduced in *Lcn2*$^{-/-}$ mice, as previously shown for *Cxcl-8* (Bachman et al., 2009). Nonetheless, reduction in the expression of *Cxcl-1* did not result in differences in the neutrophil influx observed by the pathology, likely because of redundant mechanisms of neutrophil migration to the gut (light blue bars, Fig. S4 and S5). Of note, administration of *E. coli* Nissle to *Lcn2*$^{-/-}$ mice infected with *S.* Typhimurium also ameliorated intestinal inflammation, despite the minimal differences observed in the expression of pro-inflammatory cytokines. These results may indicate that lipocalin-2 has additional immunomodulatory effects that are independent of its iron acquisition mechanisms(Bachman et al., 2009).

While we had administered *E. coli* Nissle as a therapeutic either during or after *S.* Typhimurium administration, this strain was originally isolated as a normal commensal of

the gut flora from a healthy soldier who did not acquire *Shigella* during an outbreak. To test whether colonization of mice with *E. coli* Nissle would confer protection to infection, C57BL/6 mice were administered *E. coli* Nissle three days prior to *S.* Typhimurium infection. As shown in Fig. 6C, mice that were pre-colonized with *E. coli* Nissle showed a significant reduction in colonization from *S.* Typhimurium, indicating that colonization with this probiotic offers at least partial protection to infection with a gastrointestinal pathogen.

Next, we determined the ratio of the *S.* Typhimurium colony forming units detected in the colonic content of C57BL/6 mice when *S.* Typhimurium was administered alone versus in competition with *E. coli* Nissle. Overall, there was a marked reduction (up to 445-fold) in *S.* Typhimurium fecal colonization in wild-type mice that were co-administered wild-type *E. coli* Nissle at the time of infection relative to mice that were infected with *S.* Typhimurium only, a difference which was not observed in either $Lcn2^{-/-}$ mice or wild-type mice that were administered either the *E. coli* Nissle *tonB* or the *E. coli* Nissle Δ4 mutants (Fig. 7A, C, E). Furthermore, only wild-type *E. coli* Nissle co-administered with *S.* Typhimurium to wild-type mice was able to outcompete *S.* Typhimurium by up to 195-fold (Fig. 7B, D, F). Taken together, our results show that iron acquisition in the inflamed gut is a critical mechanism for the ability of the probiotic *E. coli* Nissle to limit *Salmonella* intestinal colonization.

Discussion

E. coli Nissle was isolated from a healthy soldier during a *Shigella* outbreak in 1917 under the hypothesis that a protective commensal strain must have colonized the gut of that soldier (Nissle, 1959). In the years since, *E. coli* Nissle has had a long history of use in clinical settings to treat gastrointestinal disorders, though the molecular mechanism of its beneficial activity is not well understood (Schultz, 2008), much like all natural probiotics (reviewed in (Balakrishnan and Floch, 2012)).

Probiotics may exert their beneficial effects by either direct interaction with the host, or by competition with pathogenic species (mechanisms frequently grouped under the term “colonization resistance”; reviewed in (Lawley and Walker, 2013)). A large focus of research on probiotics has been on their interactions with the immune system rather than their competition with other microbes. Regarding *E. coli* Nissle, several studies have proposed that selective immune modulation may contribute to its activity(reviewed in (Behnsen et al., 2013; Jacobi and Malfertheiner, 2011), including the activation of γδ T cells (Guzy et al., 2008), the reduction of the secretion of pro-inflammatory cytokines in the mucosa (Grabig et al., 2006), the enhanced secretion of IgA and IgM (Cukrowska et al., 2002), and the production of tight junction proteins (Ukena et al., 2007) and human beta-defensin-2 (Wehkamp et al., 2004). While *E. coli* Nissle has been proposed to have both pro- and anti-inflammatory effects, the net result of these alterations is an overall enhancement of the mucosal barrier. Consistent with these findings, as well as with other studies that have shown *E. coli* Nissle to ameliorate the disease caused by an infection (Hockertz, 1997; Splichalova et al., 2011), we also observed a reduction in both intestinal pathology and expression of pro-inflammatory cytokines when *S.* Typhimurium-infected mice were administered *E. coli* Nissle in both a chronic and an acute model of infection, as well as when we induced inflammation independent of infection.

The two mouse models that we employed (129X1/SvJ for the chronic model; C57BL/6 for the acute model) develop intestinal inflammation when infected with *S.* Typhimurium after streptomycin treatment. However, the host response to *S.* Typhimurium is not identical in these mice because of genetic differences as well as differences in the composition of the microbiota. At present, the best studied genetic distinction is that 129X1/SvJ have a

functional *Nramp1* (*Slc11a1*) allele, which renders the mice more resistant to infection with *S*. Typhimurium. However, functional Nramp1 alone does not explain differences in the host response to *S*. Typhimurium (Brown et al., 2013), and altered expression or function of other genes (for instance, caspase-11) may also play a role (Kayagaki et al., 2011). These variances may explain why in the chronic model of infection only wild-type *E. coli* Nissle mediated a reduction of inflammation, while in the acute model of infection the reduction was independent of iron acquisition. Nevertheless, our study indicates that the mild-to-moderate reduction in intestinal inflammation only partly explains the beneficial effects of administering *E. coli* Nissle during infection.

Intestinal inflammation of infectious and non-infectious origins results in an alteration of the normal flora and a significant microbial dysbiosis, including the loss of Bacteroidetes and Firmicutes and the proliferation of Enterobacteriaceae (reviewed in (Fava and Danese, 2011; Mukhopadhya et al., 2012)). A mechanism for the proliferation of Enterobacteriaceae in the inflamed gut was recently provided by Winter and colleagues, who showed that this family can utilize host-derived nitrate for respiration (Winter et al., 2013). The fact that *E. coli* Nissle also benefits from inflammation could also explain why its anti-inflammatory effects are mild to moderate: a complete reduction of inflammation would be detrimental to its own colonization, as indicated in our mice that were pre-colonized with *E. coli* Nissle prior to *S*. Typhimurium infection. A change in the composition of the normal flora, with loss of Bacteroidetes and Firmicutes, is also observed during infection caused by *S*. Typhimurium as this pathogen has been shown to thrive during inflammation and successfully compete with the microbiota (reviewed in (Thiennimitr et al., 2012)). While the host inflammatory response limits the availability of essential nutrients, including metal ions, to invading microbes in what is known as nutritional immunity, pathogens including *Salmonella* have evolved many mechanisms to evade this response and acquire essential metals necessary to mount a successful infection(Thiennimitr et al., 2012).

Our previous studies indicated that iron uptake via salmochelin, which confers resistance to iron sequestration mediated by lipocalin-2, is essential for efficient colonization of *S*. Typhimurium (Crouch et al., 2008; Raffatellu et al., 2009). As evasion of lipocalin-2-mediated iron withholding has also been found to be essential to the virulence of many other Gram-negative enteric pathogens (Bachman et al., 2009; Caza et al., 2008; Garcia et al., 2011; Himpsl et al., 2010; Payne et al., 2006), this mechanism has come to be viewed as a virulence trait. In contrast to this trend, our research demonstrates that lipocalin-2-resistant iron acquisition is not a property unique to virulence as it is essential for the probiotic activity of *E. coli* Nissle.

Deletion of up to three iron receptors did not result in a growth defect in media supplemented with lipocalin-2 (data not shown), indicating that *E. coli* Nissle possesses redundant lipocalin-2-resistant iron uptake systems. Integrating this finding with our observation that *E. coli* Nissle outcompetes *S.* Typhimurium *in vivo* along with our previous finding that *S.* Typhimurium benefits from acquiring iron in a lipocalin-2 resistant fashion during inflammation, we hypothesized that Nissle's multiple iron uptake systems provide a competitive advantage against *S*. Typhimurium when the intestine is inflamed and, as we observed, fecal iron is significantly reduced. Consistent with our hypothesis, our results show that *E. coli* Nissle's ability to displace the highly evolved pathogen from its intestinal niche is dependent on iron acquisition. Furthermore, *E. coli* Nissle was only able to reduce the colonization of *S*. Typhimurium when lipocalin-2 was expressed. While lipocalin-2 is one of the host defenses exploited by *S.* Typhimurium to colonize the inflamed gut and compete with the microbiota, *E. coli* Nissle also subverts this host defense mechanism to thrive in the same inflamed and iron-starved environment. By scavenging for iron more effectively than *S.* Typhimurium, both with its own armament of siderophores and with its

ability to compete with *S.* Typhimurium for uptake of salmochelin, *E. coli* Nissle tips the scales back in favor of the host, effectively augmenting the host's innate immune response by acting as a surrogate of sorts for lipocalin-2. It is along these lines that we propose that *E. coli* Nissle – and possibly other beneficial components of the microbiota – may provide colonization resistance in part by boosting the host's nutritional immunity, sequestering nutrients from pathogens when the host fails to do so.

Taken together, our results show that iron acquisition in the inflamed gut is a critical mechanism for the ability of the probiotic *E. coli* Nissle to limit *Salmonella* intestinal colonization. Furthermore, we have demonstrated that this action of *E. coli* Nissle results from its resistance to lipocalin-2, previously considered a mechanism of virulence but now also seen as an essential property of a protective commensal organism. As antibiotic treatment is contraindicated for uncomplicated *Salmonella* infections due to prolongation of fecal shedding, the administration of *E. coli* Nissle may be a feasible alternative to diminish *Salmonella* colonization and ameliorate symptoms. As microbial dysbiosis is apparent in a variety of intestinal disorders (DuPont and DuPont, 2011), iron acquisition may also contribute to other probiotic actions attributed to *E. coli* Nissle. The ability of *E. coli* Nissle to withstand inflammation and outcompete a highly evolved pathogen for an essential micronutrient may be seen as a paradigm for understanding the protective actions of commensal microorganisms and a foundation upon which to build future probiotics tailored to the treatment of different diseases.

Experimental Procedures

Bacterial Strains and Culture Conditions

All strains used in this study are listed in Table S1. *S.* Typhimurium strain IR715 is a fully virulent, nalidixic acid resistant derivative of wild-type isolate ATCC 14028. *Escherichia coli* Nissle 1917 wild-type is a non-pathogenic human *E. coli* isolate that we obtained from Ulrich Sonnenborn, Ardeypharm. An IR715 derivative carrying a mutation in *iron,* and *E. coli* Nissle derivatives carrying mutations in *tonB* or *iroN*, *iutA*, *fyuA*, and *chuA* were used for this study. Mutant construction is described in the supporting information and the plasmids and primers used are detailed in Tables S1 and S2. For animal infections, all strains were grown in Miller Luria-Bertani (LB) media at 37°C with aeration overnight.

In vitro growth assays

S. Typhimurium and *E. coli* Nissle strains were tested for the ability to grow in iron limiting conditions (Nutrient Broth supplemented with 0.2 mM 2,2-Dipyridyl, Sigma) at 37°C with aeration overnight. To test lipocalin-2 sensitivity, approximately 10^3 CFU from an overnight culture were inoculated into tissue culture medium comprising DMEM/F12 (Invitrogen) plus 10% fetal bovine serum (FBS, Invitrogen) or in the same medium containing human lipocalin-2 (1μg/ml, R&D Systems) as previously published (Raffatellu et al., 2009). To compare general growth rates, a 1:1 mixture of *E. coli* Nissle wild-type and either the *tonB* or the Δ4 mutants containing 1×10^7 CFU was inoculated in M9 minimal media at 37°C with aeration. When indicated, iron (III) citrate and iron (III) sulfate were added at a final concentration of 1mM and 200 μM, respectively. CFU were enumerated by plating serial dilutions at 2h, 5h and 8h after inoculation.

Animal infections

For acute infections, female C57BL/6 (Taconic) and lipocalin-2-deficient ($Lcn2^{-/-}$) mice were orally gavaged with a dose of 10^9 CFU in 100μl of phosphate-buffered saline (PBS) 24 hours after pre-treatment with streptomycin (100μl of a 200 mg/ml solution in sterile water) (Barthel et al., 2003; Raffatellu et al., 2009). Mice were infected with either *S.* Typhimurium

alone or with a 1:1 mixture of strains as indicated. Fecal pellets were collected at 48 and 72 hours post-infection and weighed for CFU determination, homogenized in 1 ml of sterile PBS, and serial dilutions were plated on LB agar containing appropriate antibiotics. At 96 hours post-infection, mice were euthanized and the cecum was collected for isolation of mRNA and for histopathology; the liver was collected as indicated to measure hepatic gene expression. Bacteria were enumerated in the colon content on agar plates containing the appropriate antibiotics. To render all strains equally resistant to streptomycin, either pACYomega or pHP45omega (Table S1) were introduced by electroporation. When noted, the competitive indices were calculated by dividing the output ratio (*E. coli* Nissle CFU / *S.* Typhimurium CFU) by the input ratio (*E. coli* Nissle CFU / *S.* Typhimurium CFU). In some groups of mice, a single dose of 10^9 CFU of *E. coli* Nissle was administered three days prior to *S.* Typhimurium infection, as indicated. In other groups, colitis was induced by administration of dextran sodium sulfate (Wirtz et al., 2007); some of these mice were administered a single dose of 10^9 CFU *E. coli* Nissle the same day DSS treatment was started (day 1). DSS-treated mice were sacrificed at day 6 and the cecum was harvested for RNA purification and analysis.

For chronic infections, female 129X1/SvJ mice (Jackson Laboratories, Bar Harbor, ME) were orally gavaged as described above with *S.* Typhimurium 24 hours after pre-treatment with streptomycin (Lawley et al., 2008). 72 hours post-infection, groups of mice were administrated a single dose of 10^9 CFU *E. coli* Nissle wild-type or *E. coli tonB* mutant in 100 μl of LB by oral gavage. Individual mice were followed for the duration of the experiment (up to 22 days). Fecal pellets and colon contents were collected and processed as described above.

Quantitative Real-Time PCR

For analysis of gene expression by quantitative real-time PCR, total RNA was extracted from cecal and hepatic tissues with TRI Reagent (Molecular Research Center; Cincinnati, OH). For DSS-treated mice, oligo(dT) purification of mRNA was performed using the Dynabeads mRNA Purification kit (Invitrogen). Real-time PCR was performed using SYBR Green (Roche, Indianapolis, IN) and the Roche Lightcycler 480 system (Roche, Indianapolis, IN). Data were analyzed using the comparative ΔΔ-Ct method. Target gene transcription of each sample was normalized to the respective levels of mRNA *β-actin*. A list of the real-time primers used in this study is provided in Table S3.

Histopathology

Tissue samples were fixed in formalin, processed according to standard procedures for paraffin embedding, sectioned at 5 μm, and stained with hematoxylin and eosin. Blinded examination by a board-certified pathologist was used to score the pathology of cecal samples using previously published methods (Barthel et al., 2003; Raffatellu et al., 2009). Each section was evaluated for the presence of neutrophils, mononuclear infiltrate, submucosal edema, surface erosions, inflammatory exudates, and cryptitis. Inflammatory changes were scored from 0 to 4 according to the following scale: 0 = none; 1 = low; 2 = moderate; 3 = high; 4 = extreme. The inflammation score for each mouse was calculated by adding the score for each parameter and was interpreted as follows: 0–2 = within normal limit; 3–5 = mild; 6–8 = moderate; 8+ = severe.

Measurement of Iron in Fecal Samples by ICP-MS

The amount of iron in mouse fecal samples was measured by ICP-MS as described previously (Corbin et al., 2008; Liu et al., 2012) and is detailed in the Supplemental Experimental Procedures.

Statistical analysis

Differences between treatment groups were analyzed by ANOVA followed by Student's *t* test. A *P* value equal to or below 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to acknowledge Ulrich Sonnenborn and Ardeypharm for the gift of the *E. coli* Nissle wild-type strain, Sean-Paul Nuccio for help with editing the manuscript, Takeshi Haneda and Andreas Bäumler for the gift of plasmids, Eric Skaar and Russell Gerards for their help with ICP-MS, Alfredo Torres and Harry Mobley for their help with mutant construction, Elizabeth Nolan for helpful discussions, and Kelly Smith for the original gift of $Lcn2^{-/-}$ mice. This work was supported by Public Health Service Grants AI083663 (to M.R.) and AI77629 (to F.C.F.), and an IDSA ERF/NIFID Astellas Young Investigator Award to M.R.. J.Z.L. was supported by the NIH Immunology Research Training Grant T32 AI60573 and then by a predoctoral fellowship from the American Heart Association.

References

Andrews NC, Schmidt PJ. Iron homeostasis. Annu Rev Physiol. 2007; 69:69–85. [PubMed: 17014365]

Bachman MA, Miller VL, Weiser JN. Mucosal lipocalin 2 has pro-inflammatory and iron-sequestering effects in response to bacterial enterobactin. PLoS Pathog. 2009; 5:e1000622. [PubMed: 19834550]

Balakrishnan M, Floch MH. Prebiotics, probiotics and digestive health. Curr Opin Clin Nutr Metab Care. 2012; 15:580–585. [PubMed: 23037903]

Barman M, Unold D, Shifley K, Amir E, Hung K, Bos N, Salzman N. Enteric salmonellosis disrupts the microbial ecology of the murine gastrointestinal tract. Infect Immun. 2008; 76:907–915. [PubMed: 18160481]

Barthel M, Hapfelmeier S, Quintanilla-Martinez L, Kremer M, Rohde M, Hogardt M, Pfeffer K, Russmann H, Hardt WD. Pretreatment of mice with streptomycin provides a *Salmonella enterica* serovar Typhimurium colitis model that allows analysis of both pathogen and host. Infect Immun. 2003; 71:2839–2858. [PubMed: 12704158]

Behnsen J, Deriu E, Sassone-Corsi M, Raffatellu M. Probiotics: properties, examples, and specific applications. Cold Spring Harb Perspect Med. 2013; 3:a010074. [PubMed: 23457295]

Berger T, Togawa A, Duncan GS, Elia AJ, You-Ten A, Wakeham A, Fong HE, Cheung CC, Mak TW. Lipocalin 2-deficient mice exhibit increased sensitivity to *Escherichia coli* infection but not to ischemia-reperfusion injury. Proc Natl Acad Sci U S A. 2006; 103:1834–1839. [PubMed: 16446425]

Braun V, Hantke K. Recent insights into iron import by bacteria. Curr Opin Chem Biol. 2011; 15:328–334. [PubMed: 21277822]

Brown DE, Libby SJ, Moreland SM, McCoy MW, Brabb T, Stepanek A, Fang FC, Detweiler CS. *Salmonella enterica* Causes More Severe Inflammatory Disease in C57/BL6 Nramp1G169 Mice Than Sv129S6 Mice. Vet Pathol. 2013

Cassat JE, Skaar EP. Iron in infection and immunity. Cell Host Microbe. 2013; 13:509–519. [PubMed: 23684303]

Caza M, Lepine F, Milot S, Dozois CM. Specific roles of the *iroBCDEN* genes in virulence of an avian pathogenic *Escherichia coli* O78 strain and in production of salmochelins. Infect Immun. 2008; 76:3539–3549. [PubMed: 18541653]

Corbin BD, Seeley EH, Raab A, Feldmann J, Miller MR, Torres VJ, Anderson KL, Dattilo BM, Dunman PM, Gerads R, et al. Metal chelation and inhibition of bacterial growth in tissue abscesses. Science. 2008; 319:962–965. [PubMed: 18276893]

Crouch ML, Castor M, Karlinsey JE, Kalhorn T, Fang FC. Biosynthesis and IroC-dependent export of the siderophore salmochelin are essential for virulence of *Salmonella enterica* serovar Typhimurium. Molecular microbiology. 2008; 67:971–983. [PubMed: 18194158]

Cukrowska B, LodInova-ZadnIkova R, Enders C, Sonnenborn U, Schulze J, Tlaskalova-Hogenova H. Specific proliferative and antibody responses of premature infants to intestinal colonization with nonpathogenic probiotic *E. coli* strain Nissle 1917. Scand J Immunol. 2002; 55:204–209. [PubMed: 11896937]

DuPont AW, DuPont HL. The intestinal microbiota and chronic disorders of the gut. Nat Rev Gastroenterol Hepatol. 2011; 8:523–531. [PubMed: 21844910]

Fava F, Danese S. Intestinal microbiota in inflammatory bowel disease: friend of foe? World J Gastroenterol. 2011; 17:557–566. [PubMed: 21350704]

Fischbach MA, Lin H, Liu DR, Walsh CT. How pathogenic bacteria evade mammalian sabotage in the battle for iron. Nature chemical biology. 2006a; 2:132–138.

Fischbach MA, Lin H, Zhou L, Yu Y, Abergel RJ, Liu DR, Raymond KN, Wanner BL, Strong RK, Walsh CT, et al. The pathogen-associated *iroA* gene cluster mediates bacterial evasion of lipocalin 2. Proceedings of the National Academy of Sciences of the United States of America. 2006b; 103:16502–16507. [PubMed: 17060628]

Flo TH, Smith KD, Sato S, Rodriguez DJ, Holmes MA, Strong RK, Akira S, Aderem A. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. Nature. 2004; 432:917–921. [PubMed: 15531878]

Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood. 2003; 102:783–788. [PubMed: 12663437]

Garcia EC, Brumbaugh AR, Mobley HL. Redundancy and specificity of *Escherichia coli* iron acquisition systems during urinary tract infection. Infect Immun. 2011; 79:1225–1235. [PubMed: 21220482]

Grabig A, Paclik D, Guzy C, Dankof A, Baumgart DC, Erckenbrecht J, Raupach B, Sonnenborn U, Eckert J, Schumann RR, et al. *Escherichia coli* strain Nissle 1917 ameliorates experimental colitis via toll-like receptor 2- and toll-like receptor 4-dependent pathways. Infection and immunity. 2006; 74:4075–4082. [PubMed: 16790781]

Grozdanov L, Raasch C, Schulze J, Sonnenborn U, Gottschalk G, Hacker J, Dobrindt U. Analysis of the genome structure of the nonpathogenic probiotic *Escherichia coli* strain Nissle 1917. Journal of bacteriology. 2004; 186:5432–5441. [PubMed: 15292145]

Guzy C, Paclik D, Schirbel A, Sonnenborn U, Wiedenmann B, Sturm A. The probiotic *Escherichia coli* strain Nissle 1917 induces gammadelta T cell apoptosis via caspase- and FasL-dependent pathways. Int Immunol. 2008; 20:829–840. [PubMed: 18448456]

Hantke K, Nicholson G, Rabsch W, Winkelmann G. Salmochelins, siderophores of *Salmonella enterica* and uropathogenic *Escherichia coli* strains, are recognized by the outer membrane receptor IroN. Proc Natl Acad Sci U S A. 2003; 100:3677–3682. [PubMed: 12655053]

Henker J, Laass M, Blokhin BM, Bolbot YK, Maydannik VG, Elze M, Wolff C, Schulze J. The probiotic *Escherichia coli* strain Nissle 1917 (EcN) stops acute diarrhoea in infants and toddlers. European journal of pediatrics. 2007; 166:311–318. [PubMed: 17287932]

Himpsl SD, Pearson MM, Arewang CJ, Nusca TD, Sherman DH, Mobley HL. Proteobactin and a yersiniabactin-related siderophore mediate iron acquisition in *Proteus mirabilis*. Mol Microbiol. 2010; 78:138–157. [PubMed: 20923418]

Hockertz S. Augmentation of host defence against bacterial and fungal infections of mice pretreated with the non-pathogenic *Escherichia coli* strain Nissle 1917. Arzneimittelforschung. 1997; 47:793–796. [PubMed: 9239462]

Jacobi CA, Malfertheiner P. *Escherichia coli* Nissle 1917 (Mutaflor): new insights into an old probiotic bacterium. Dig Dis. 2011; 29:600–607. [PubMed: 22179217]

Kayagaki N, Warming S, Lamkanfi M, Vande Walle L, Louie S, Dong J, Newton K, Qu Y, Liu J, Heldens S, et al. Non-canonical inflammasome activation targets caspase-11. Nature. 2011; 479:117–121. [PubMed: 22002608]

Kruis W, Fric P, Pokrotnieks J, Lukas M, Fixa B, Kascak M, Kamm MA, Weismueller J, Beglinger C, Stolte M, et al. Maintaining remission of ulcerative colitis with the probiotic *Escherichia coli* Nissle 1917 is as effective as with standard mesalazine. Gut. 2004; 53:1617–1623. [PubMed: 15479682]

Lawley TD, Bouley DM, Hoy YE, Gerke C, Relman DA, Monack DM. Host transmission of *Salmonella enterica* serovar Typhimurium is controlled by virulence factors and indigenous intestinal microbiota. Infect Immun. 2008; 76:403–416. [PubMed: 17967858]

Lawley TD, Walker AW. Intestinal colonization resistance. Immunology. 2013; 138:1–11. [PubMed: 23240815]

Liu JZ, Jellbauer S, Poe AJ, Ton V, Pesciaroli M, Kehl-Fie TE, Restrepo NA, Hosking MP, Edwards RA, Battistoni A, et al. Zinc sequestration by the neutrophil protein calprotectin enhances *Salmonella* growth in the inflamed gut. Cell Host Microbe. 2012; 11:227–239. [PubMed: 22423963]

Lodinova-Zadnikova R, Sonnenborn U. Effect of preventive administration of a nonpathogenic *Escherichia coli* strain on the colonization of the intestine with microbial pathogens in newborn infants. Biology of the neonate. 1997; 71:224–232. [PubMed: 9129791]

Lupp C, Robertson ML, Wickham ME, Sekirov I, Champion OL, Gaynor EC, Finlay BB. Host-mediated inflammation disrupts the intestinal microbiota and promotes the overgrowth of Enterobacteriaceae. Cell Host Microbe. 2007; 2:204. [PubMed: 18030708]

Mollenbrink M, Bruckschen E. Treatment of chronic constipation with physiologic *Escherichia coli* bacteria. Results of a clinical study of the effectiveness and tolerance of microbiological therapy with the *E. coli* Nissle 1917 strain (Mutaflor). Med Klin (Munich). 1994; 89:587–593. [PubMed: 7815986]

Mukhopadhya I, Hansen R, El-Omar EM, Hold GL. IBD-what role do Proteobacteria play? Nat Rev Gastroenterol Hepatol. 2012; 9:219–230. [PubMed: 22349170]

Muller SI, Valdebenito M, Hantke K. Salmochelin, the long-overlooked catecholate siderophore of *Salmonella*. Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine. 2009; 22:691–695.

Nissle A. Explanations of the significance of colonic dysbacteria & the mechanism of action of *E. coli* therapy (mutaflor). Medizinische. 1959; 4:1017–1022. [PubMed: 13673841]

Payne SM, Wyckoff EE, Murphy ER, Oglesby AG, Boulette ML, Davies NM. Iron and pathogenesis of *Shigella*: iron acquisition in the intracellular environment. Biometals. 2006; 19:173–180. [PubMed: 16718602]

Pressler U, Staudenmaier H, Zimmermann L, Braun V. Genetics of the iron dicitrate transport system of *Escherichia coli*. J Bacteriol. 1988; 170:2716–2724. [PubMed: 2836368]

Raffatellu M, George MD, Akiyama Y, Hornsby MJ, Nuccio SP, Paixao TA, Butler BP, Chu H, Santos RL, Berger T, et al. Lipocalin-2 resistance confers an advantage to *Salmonella enterica* serotype Typhimurium for growth and survival in the inflamed intestine. Cell host & microbe. 2009; 5:476–486. [PubMed: 19454351]

Raymond KN, Dertz EA, Kim SS. Enterobactin: an archetype for microbial iron transport. Proc Natl Acad Sci U S A. 2003; 100:3584–3588. [PubMed: 12655062]

Schultz M. Clinical use of *E. coli* Nissle 1917 in inflammatory bowel disease. Inflamm Bowel Dis. 2008; 14:1012–1018. [PubMed: 18240278]

Splichalova A, Trebichavsky I, Rada V, Vlkova E, Sonnenborn U, Splichal I. Interference of *Bifidobacterium choerinum* or *Escherichia coli* Nissle 1917 with *Salmonella* Typhimurium in gnotobiotic piglets correlates with cytokine patterns in blood and intestine. Clin Exp Immunol. 2011; 163:242–249. [PubMed: 21155989]

Stecher B, Robbiani R, Walker AW, Westendorf AM, Barthel M, Kremer M, Chaffron S, Macpherson AJ, Buer J, Parkhill J, et al. *Salmonella enterica* serovar typhimurium exploits inflammation to compete with the intestinal microbiota. PLoS biology. 2007; 5:2177–2189. [PubMed: 17760501]

Thiennimitr P, Winter SE, Bäumler AJ. *Salmonella*, the host and its microbiota. Curr Opin Microbiol. 2012; 15:108–114. [PubMed: 22030447]

Thiennimitr P, Winter SE, Winter MG, Xavier MN, Tolstikov V, Huseby DL, Sterzenbach T, Tsolis RM, Roth JR, Bäumler AJ. Intestinal inflammation allows *Salmonella* to use ethanolamine to compete with the microbiota. Proc Natl Acad Sci U S A. 2011; 108:17480–17485. [PubMed: 21969563]

Ukena SN, Singh A, Dringenberg U, Engelhardt R, Seidler U, Hansen W, Bleich A, Bruder D, Franzke A, Rogler G, et al. Probiotic *Escherichia coli* Nissle 1917 inhibits leaky gut by enhancing mucosal integrity. PLoS One. 2007; 2:e1308. [PubMed: 18074031]

Wehkamp J, Harder J, Wehkamp K, Wehkamp-von Meissner B, Schlee M, Enders C, Sonnenborn U, Nuding S, Bengmark S, Fellermann K, et al. NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a novel effect of a probiotic bacterium. Infection and immunity. 2004; 72:5750–5758. [PubMed: 15385474]

Weinberg ED. Iron withholding: a defense against infection and neoplasia. Physiol Rev. 1984; 64:65–102. [PubMed: 6420813]

Winter SE, Thiennimitr P, Winter MG, Butler BP, Huseby DL, Crawford RW, Russell JM, Bevins CL, Adams LG, Tsolis RM, et al. Gut inflammation provides a respiratory electron acceptor for *Salmonella*. Nature. 2010; 467:426–429. [PubMed: 20864996]

Winter SE, Winter MG, Xavier MN, Thiennimitr P, Poon V, Keestra AM, Laughlin RC, Gomez G, Wu J, Lawhon SD, et al. Host-derived nitrate boosts growth of *E. coli* in the inflamed gut. Science. 2013; 339:708–711. [PubMed: 23393266]

Wirtz S, Neufert C, Weigmann B, Neurath MF. Chemically induced mouse models of intestinal inflammation. Nat Protoc. 2007; 2:541–546. [PubMed: 17406617]

Highlights

- Probiotic *E. coli* Nissle reduces *Salmonella* intestinal colonization and persistence
- *E. coli* Nissle outcompetes *Salmonella* for iron in the inflamed gut
- Specialized iron transporters are essential for *E. coli* Nissle probiotic activity
- *E. coli* Nissle overcomes lipocalin-2-mediated iron sequestration

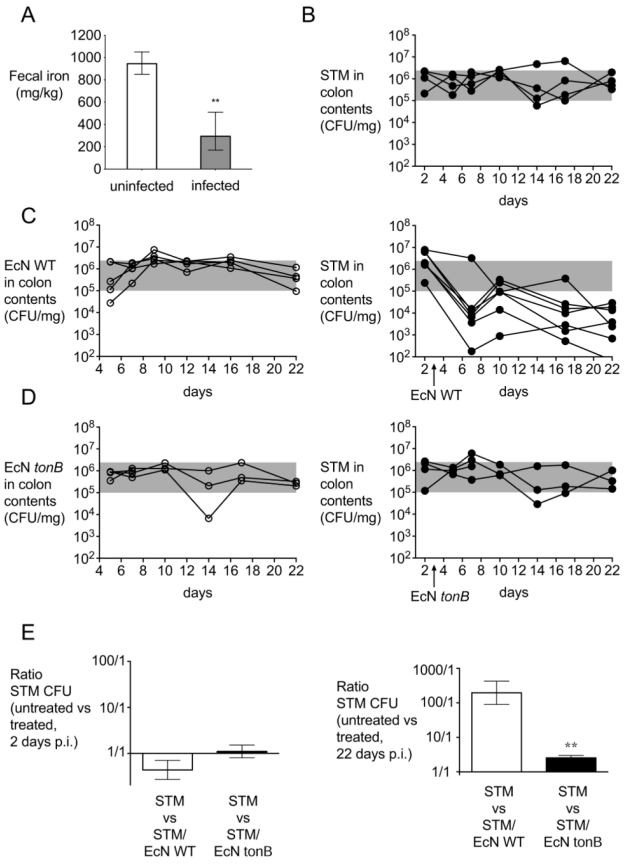

Figure 1. Probiotic *E. coli* Nissle 1917 reduces *S*. Typhimurium fecal shedding

(**A**) The concentration of iron in fecal samples collected from mock-infected (n = 4) or *S*. Typhimurium-infected (n = 4) C57BL/6 mice four days post-infection. Bars represent geometric means ± standard deviation. (**B–E**) 129X1/SvJ mice were infected with *S*. Typhimurium and either untreated (**B**) or treated with one dose of *E. coli* Nissle wild-type (**C**) or *tonB* mutant (**D**) three days after infection. *S*. Typhimurium (black circles), *E. coli* Nissle wild-type or *tonB* mutant (white circles) were enumerated in the colonic contents. (**E**) Ratio of colony forming units (CFU) recovered from fecal samples of mice infected with *S*. Typhimurium that were untreated compared with mice treated with one dose of either *E. coli* Nissle wild-type or *tonB* mutant three days after infection. Ratios 2 days after infection (i.e., 1 day before treatment) and 22 days after infection are shown. Bars represent geometric means ± standard deviation. Data are representative of n=2 experiments. STM=*S*. Typhimurium; EcN=*E. coli* Nissle. Significant difference is indicated by ** (*P* value ≤ 0.01). (See also Figure S1 and Table S1).

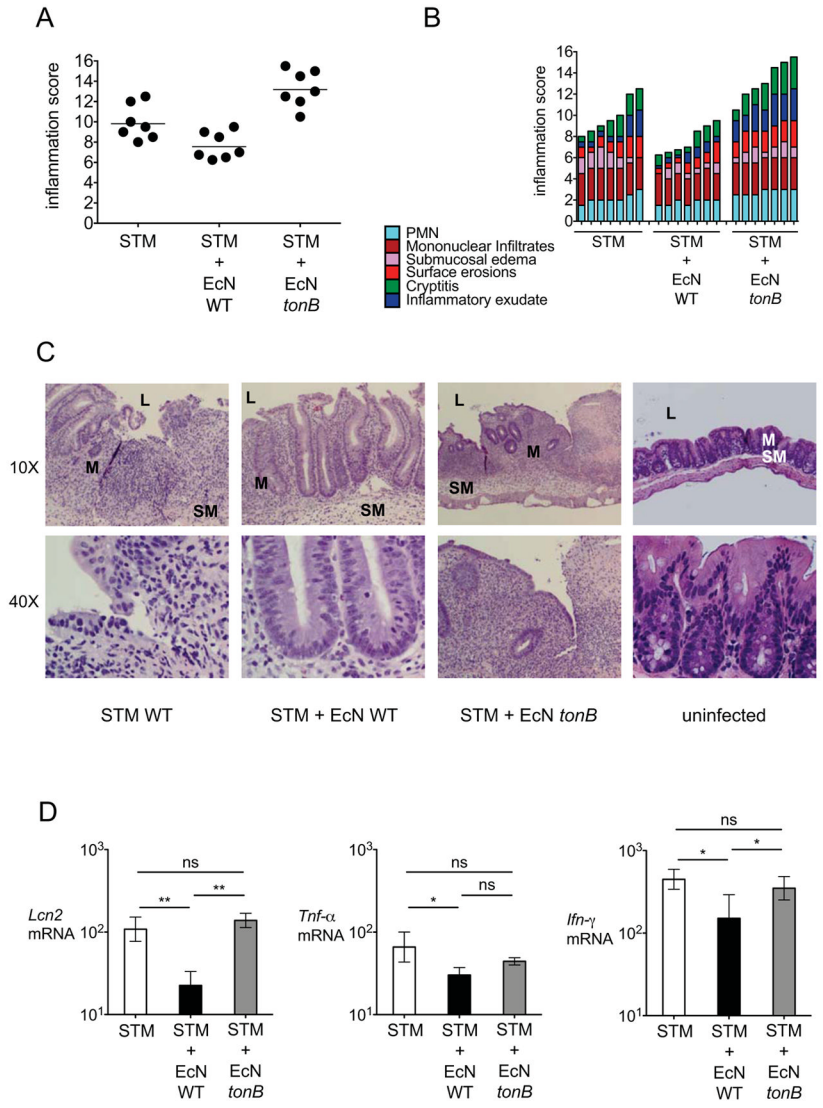

Figure 2. Intestinal host response in mice with persistent *S*. Typhimurium infection

129X1/SvJ mice were infected with *S*. Typhimurium and were either left untreated or were treated with one dose of *E. coli* Nissle wild-type or *tonB* mutant three days after infection. Cecal samples were collected at 6 or 22 days after infection. (**A**) Blinded histopathology scores of cecal samples at 22 days after infection. The score of individual mice (circles) and the geometric mean for each group (bars) are indicated. (**B**) A detailed scoring for the animals shown in (**A**) is provided. Each stacked column represents an individual mouse. (**C**) H&E stained sections from representative animals for each group. Although *E. coli* Nissle wild-type had no effect on inflammatory cell infiltration, mucosal integrity (assessed by cryptitis and surface erosions) was modestly improved. (D) Transcript levels of *Lcn2, Tnf-α* and *Inf-γ* were determined in the ceca of 129X1/SvJ mice infected with *S*. Typhimurium (white bars), infected with *S*. Typhimurium and treated with either one dose of *E. coli* Nissle wild-type (black bars) or *tonB* mutant (gray bars) 3 days after infection. Samples were collected 6 days after infection. Data are expressed as fold-increase over mock-infected mice. Bars represent the geometric means ± standard deviation. STM=*S*. Typhimurium; EcN=*E. coli* Nissle; L; lumen, M, mucosa, SM; submucosa. Significant difference is indicated by * (*P* value ≤ 0.05) or ** (*P* value ≤ 0.01).

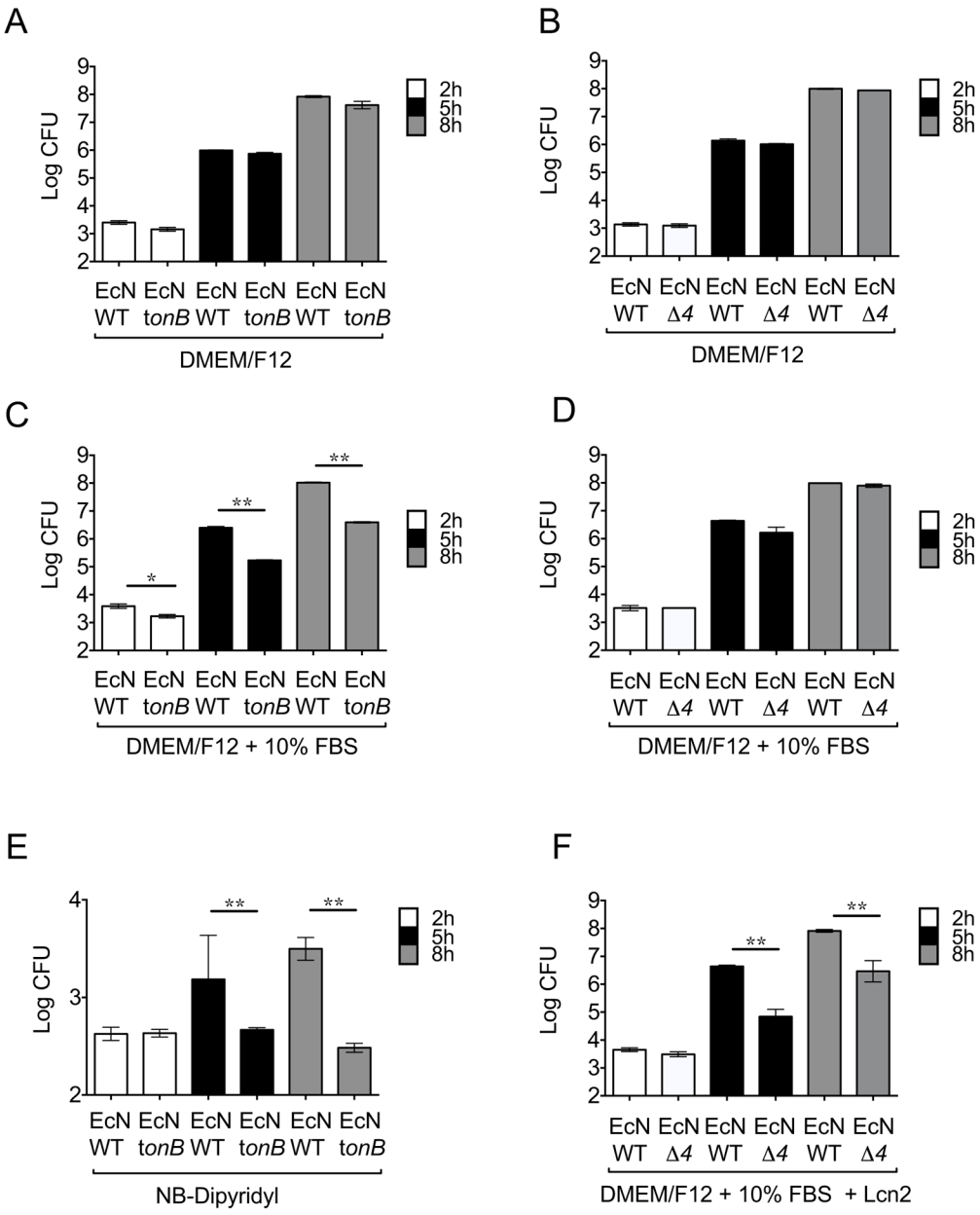

Figure 3. Growth of *E. coli* Nissle 1917 strains in iron-rich and iron-limited media

Growth of E. coli Nissle wild-type and the mutants in iron uptake *tonB* or *iroN fuyA iutA chuA* (Δ4) was determined. (**A,C,E**) Growth of *E. coli* Nissle wild-type and the *tonB* mutant in DMEM/F12 (**A**) or DMEM/F12 supplemented with 10% fetal bovine serum (**C**) or nutrient broth (NB) supplemented with Dipyridyl (**E**). (**B,D,F**) Growth of *E. coli* Nissle wild-type and the Δ4 mutant in DMEM/F12 (**B**) or DMEM/F12 supplemented with 10% fetal bovine serum with the absence (**D**) or presence (**F**) of 1μg/ml lipocalin-2 (Lcn2). Bacteria were enumerated at 2h, 5h, and 8h after inoculation. Bars represent the geometric means ± standard deviation of at least three experiments. STM=*S.* Typhimurium; EcN=*E. coli* Nissle. Significant differences are indicated by * (*P* value ≤ 0.05) or ** (*P* value ≤ 0.01). (See also Figure S2 and Table S2)

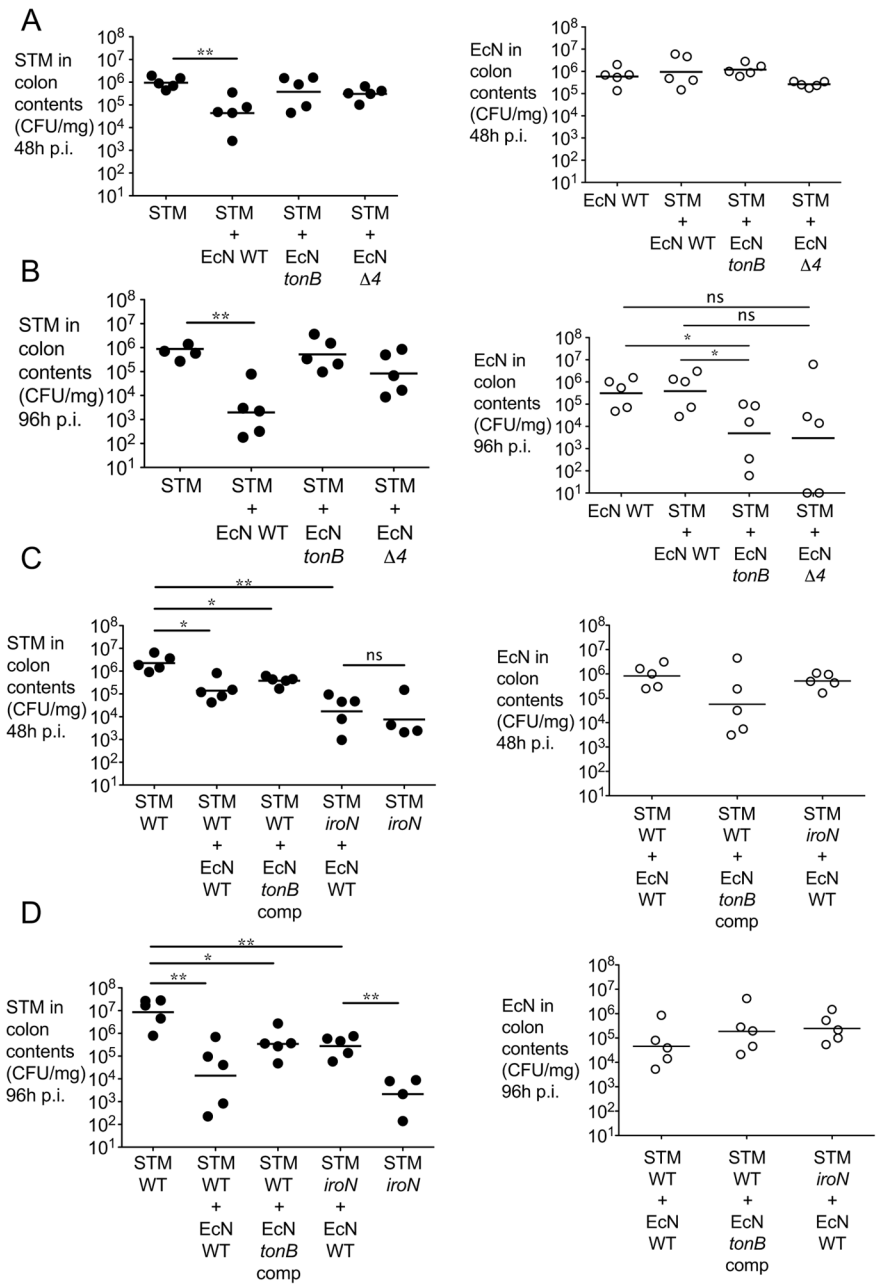

Figure 4. *E. coli* Nissle 1917 requires iron uptake systems to reduce *S.* Typhimurium intestinal colonization

(**A,B**) C57BL/6 mice were infected with *S.* Typhimurium alone or co-administered with either wild-type *E. coli* Nissle, the *tonB* mutant or the *iroN fuyA iutA chuA* mutant (Δ4). (**C,D**) C57BL/6 mice were infected with *S.* Typhimurium alone (wild-type or *iroN* mutant) or co-administered with either wild-type *E. coli* Nissle, the *tonB* mutant or the *tonB* mutant complemented *in trans* when indicated. CFU in colonic contents were enumerated at 48h (**A,C**) and 96h (**B,D**) after infection. *S.* Typhimurium (black circles) and *E. coli* Nissle (white circles) counts are shown. Representative experiments of n=2 are shown. STM=*S.* Typhimurium; EcN=*E. coli* Nissle. Significant difference is indicated by * (P value ≤ 0.05) or ** (P value ≤ 0.01). (See also Figure S3)

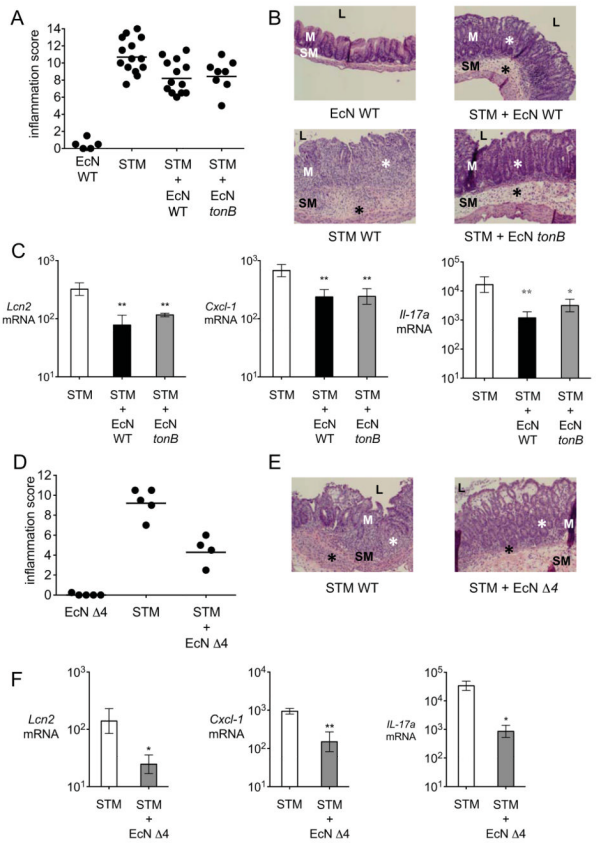

Figure 5. *E. coli* Nissle 1917 ameliorates intestinal inflammation during acute *S*. Typhimurium infection

(**A**) Blinded histopathology scores of cecal samples 4 days after infection of C57BL/6 mice administered wild-type *E. coli* Nissle, *S*. Typhimurium, or a mixture of *S*. Typhimurium and *E. coli* Nissle wild-type or *tonB* mutant. Scores of individual mice (circles) and geometric means for each group (bars) are indicated. (**B**) H&E stained sections from representative animals for each group. *E. coli* Nissle wild-type or *tonB* co-administration with *S*. Typhimurium reduced the density of inflammatory infiltrates (black asterisks) and the degree of crypt injury (white asterisks), compared to *S*. Typhimurium infection alone. (**C**) Transcript levels of *Lcn2, Cxcl-1* and *Il-17a* were determined in the ceca of C57BL/6 mice infected with *S*. Typhimurium (white bars), a mixture of *S*. Typhimurium and wild-type *E. coli* Nissle (black bars), or a mixture of *S*. Typhimurium and *E. coli* Nissle *tonB* (gray bars). Data are expressed as fold-increase over mock-infected mice. Bars represent the geometric means ± standard deviation. (**D**) Blinded histopathology scores of cecal samples 4 days after infection of mice administered *E. coli* Nissle *iroN fuyA iutA chuA* (Δ4), *S*. Typhimurium, or a mixture of *S*. Typhimurium and *E. coli* Nissle Δ4. Scores of individual mice (circles) and geometric means for each group (bars) are indicated. (**E**) H&E stained sections from representative animals from each group. *E. coli* Nissle Δ4 co-administration with *S*. Typhimurium greatly reduced chronic inflammatory infiltrates (black asterisks) and the degree of crypt injury (white asterisks), compared to *S*. Typhimurium infection alone. (**F**) Transcript levels of *Lcn2, Cxcl-1* and *Il-17a* were determined in the ceca of mice infected with *S*. Typhimurium (white bars) or a mixture of *S*. Typhimurium and *E. coli* Nissle Δ4 (gray bars). Data are expressed as fold-increase over mock-infected mice. Bars represent geometric means ± standard deviation. STM=*S*. Typhimurium; EcN=*E. coli* Nissle. L;

lumen, M, mucosa, SM; submucosa. Significant difference is indicated by * (P value ≤ 0.05) or ** (P value ≤ 0.01). (See also Figure S4 and Table S3).

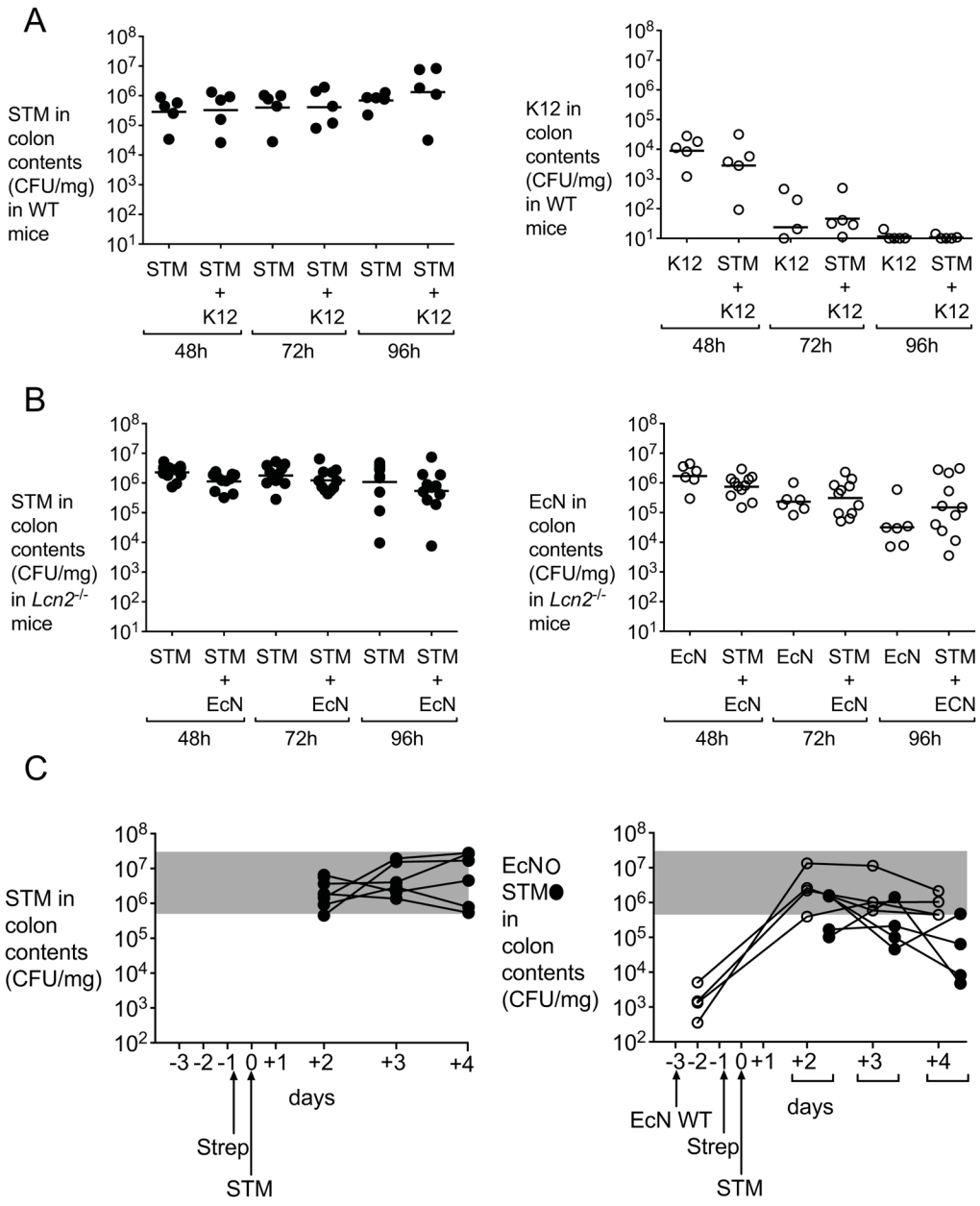

Figure 6. *E. coli* Nissle 1917 reduction of *S*. Typhimurium intestinal colonization requires functional lipocalin-2 and is independent of the time of administration

(**A**) C57BL/6 mice were infected with *S*. Typhimurium alone or co-administered with commensal *E. coli* K-12. CFU in colonic contents were enumerated at 48h, 72h and 96h post-infection. STM (black circles) and *E. coli* K-12 (white circles) counts are shown. (**B**) C57BL/6 *Lcn2*$^{-/-}$ mice were infected with *S*. Typhimurium alone or co-administered with wild-type *E. coli* Nissle. CFU in colonic contents were enumerated at 48h, 72h and 96h post-infection. *S*. Typhimurium (black circles) and *E. coli* Nissle (white circles) counts are shown. (**C**) C57BL/6 mice were administered a single dose of *E. coli* Nissle wild-type or mock, three days before being infected with *S*. Typhimurium. Streptomycin treatment was performed one day prior to *S*. Typhimurium infection. CFU in colonic contents were enumerated at 2, 3 and 4 days post-infection. *S*. Typhimurium (black circles) and *E. coli*

Nissle (white circles) counts are shown. K12=*E. coli* K12; STM=*S.* Typhimurium; EcN=*E. coli* Nissle. (See also Figure S5).

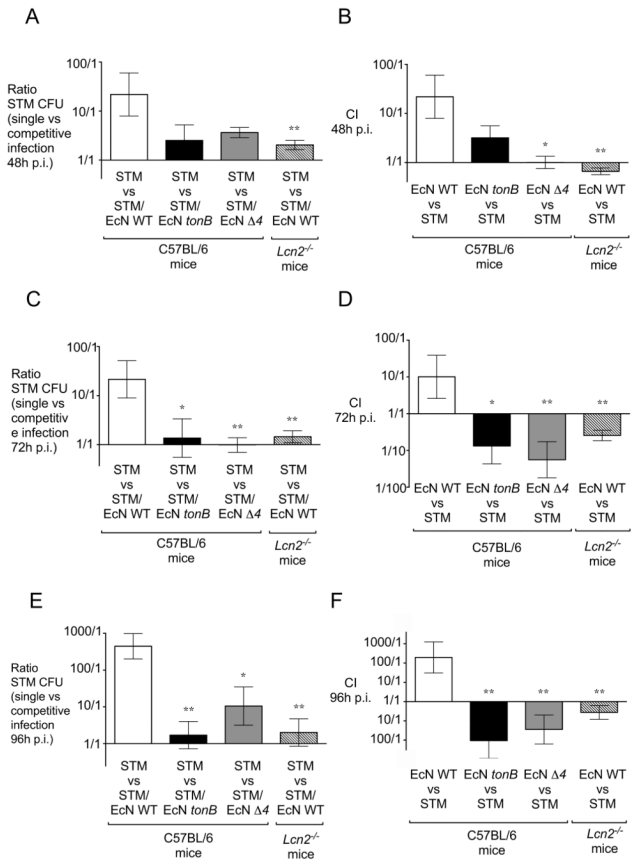

Figure 7. Ratios of *S*. Typhimurium and *E. coli* Nissle in the acute colitis model

Ratio of the CFU recovered from the fecal samples of mice infected with *S.* Typhimurium that were untreated in comparison with mice that were administered one dose of either *E. coli* Nissle wild-type, the *tonB* mutant, or the *iroN fuyA iutA chuA* (Δ4) mutant at the time of infection. (**A, C, E**) Ratio of *S.* Typhimurium CFU at 48 hours (**A**), 72 hours (**C**), 96 hours (**E**) post infection. (**B, D, F**) Competitive index (CI) in the indicated mixed infection was calculated by dividing the output ratio (*E. coli* Nissle CFU / *S.* Typhimurium CFU in the colonic contents of mice at the indicated time points by the input ratio (*E. coli* Nissle CFU / *S.* Typhimurium CFU). CI of the indicated *E. coli* Nissle strain versus *S.* Typhimurium at 48 hours (**B**), 72 hours (**D**), 96 hours (**F**) post infection. Bars represent geometric means ± standard deviation. Significant difference is indicated by * (*P* value ≤ 0.05) or ** (*P* value ≤ 0.01).