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Use of Attenuated but Metabolically Competent *Salmonella* as a Probiotic To Prevent or Treat *Salmonella* Infection

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***Salmonella enterica* is among the most burdensome of foodborne disease agents. There are over 2,600 serovars that cause a range of disease manifestations ranging from enterocolitis to typhoid fever. While there are two vaccines in use in humans to protect against typhoid fever, there are none that prevent enterocolitis. If vaccines preventing enterocolitis were to be developed, they would likely protect against only one or a few serovars. In this report, we tested the hypothesis that probiotic organisms could compete for the preferred nutrient sources of *Salmonella* and thus prevent or treat infection. To this end, we added the *fra* locus, which encodes a utilization pathway for the *Salmonella*-specific nutrient source fructose-asparagine (F-Asn), to the probiotic bacterium *Escherichia coli* Nissle 1917 (Nissle) to increase its ability to compete with *Salmonella* in mouse models. We also tested a metabolically competent, but avirulent, *Salmonella enterica* serovar Typhimurium mutant for its ability to compete with wild-type *Salmonella*. The modified Nissle strain became more virulent and less able to protect against *Salmonella* in some instances. On the other hand, the modified *Salmonella* strain was safe and effective in preventing infection with wild-type *Salmonella*. While we tested for efficacy only against *Salmonella* Typhimurium, the modified *Salmonella* strain may be able to compete metabolically with most, if not all, *Salmonella* serovars, representing a novel approach to control of this pathogen.**

Salmonella infections are among the three most common foodborne infections in the United States and are the leading cause of hospitalization and death (1). *Salmonella enterica* subsp. *enterica* includes over 1,500 serovars that can be broadly classified into two pathovars, the gastrointestinal and the extraintestinal (2). The gastrointestinal pathovar consists of serovars that have a broad host range and robust pathways for anaerobic metabolism. The extraintestinal pathovar consists of more host-restricted serovars that cause systemic disease, i.e., typhoid fever (3, 4). The extraintestinal serovars are undergoing genome reduction as they lose host range determinants and the ability to respire anaerobically (2, 5). The gastrointestinal serovars *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis are the most medically significant serovars in the United States. They can infect a remarkably broad range of host species, including a large number of different animals and even plants (6–9). In humans, *Salmonella* serovar Typhimurium causes an acute enterocolitis characterized by an inflammatory diarrhea and fever (10–12). In rare cases, this is followed by reactive arthritis (13, 14). Another form of *Salmonella* disease, invasive nontyphoidal salmonellosis (iNTS), is emerging, especially in Africa (15, 16). This disease is associated with malaria infection in children and HIV infection in adults (17–20). *Salmonella* Typhimurium and *Salmonella* Enteritidis are the most common serovars associated with iNTS. Unfortunately, there are no vaccines for the gastrointestinal serovars (21, 22). Antibiotics are not recommended for uncomplicated cases of *Salmonella*-mediated enterocolitis but are used to treat the very young or elderly or when there are complications or invasive disease. However, multiple drug resistance is prevalent and increasing (15, 23). Novel therapeutic approaches are needed for the gastrointestinal salmonelloses.

Infection of *slc11A1* mutant mice (formerly known as *Nramp1*), such as C57BL/6 or BALB/c, by *Salmonella* serovar Typhimurium is often used as a surrogate model for *Salmonella* serovar Typhi infection of humans. This is because the intestine shows little or no inflammation and there is no diarrhea but there is a systemic lethal infection. However, the lack of inflammation makes this a poor model for the natural disease caused by *Salmonella* Typhimurium, which is inflammatory diarrhea. It has been known for decades that the normal intestinal microbiota protects against systemic *Salmonella* infection, referred to as colonization resistance. For instance, the 50% lethal dose (LD_{50}) for *Salmonella* Typhimurium in the BALB/c or C57BL/6 mouse decreases from 10^6 CFU for a mouse with normal microbiota to less than 10 CFU for a germfree mouse or a mouse pretreated with streptomycin (Strep treated [24, 25]). More recently, it was determined that the gastrointestinal tracts of germfree and Strep-treated mice become inflamed by *Salmonella* Typhimurium, mimicking the human disease (26). The germfree and Strep-treated murine models are now widely used to study *Salmonella* Typhimurium-induced inflammatory diarrhea (26–35). The Strep-treated model has the advantage that the mice have a normal immune system before disruption of the microbiota with streptomycin. The germfree mice have the advantage that defined microbial communities can be created, or they can accept transplants of microbiota from different animals or humans (35–37). Germfree mice are highly susceptible to intestinal infections, and we use them in this study to gauge the safety of our proposed probiotics. The newest inflammation

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TABLE 1 Strains and plasmids

Strain or plasmid	Genotype	Reference or source
Escherichia coli strains		
Nissle 1917	*E. coli* Nissle, serotype O6:K5:H1	62
14028	Wild-type *Salmonella enterica* serovar Typhimurium	American Type Culture Collection
ASD100	14028 Δ(*ssrB-ssaU*)*1*::Kan	Lambda Red mutation of SPI2 using primers BA2558 and BA2559
ASD199	14028 Δ(*avrA-invH*)*1* Δ(*ssrB-ssaU*)*1*::Kan	Δ(*ssrB-ssaU*)*1*::Kan mutation from ASD100 transduced into YD039
ASD200	14028 Δ(*avrA-invH*)*1* Δ(*ssrB-ssaU*)*1*	Kan cassette in ASD199 was flipped out using pCP20
ASD201	14028 Δ(*avrA-invH*)*1* Δ(*ssrB-ssaU*)*1* Δ(*fraR-fraBDAE*)4::Kan	Δ(*fraR-fraBDAE*)4::Kan mutation from CS1005 was transduced into ASD200
ASD9000	*E. coli* Nissle 1917 plus pWSK29 (Amp^r)	*E. coli* Nissle 1917 electroporated with empty vector pWSK29
ASD9010	*E. coli* Nissle 1917 plus pASD5006 (Amp^r)	*E. coli* Nissle 1917 electroporated with pASD5006
CS1005	14028 Δ(*fraR-fraBDAE*)4::Kan	Lambda Red mutation of *fra* island using primers BA2515 and BA2538
JLD1214	14028 IG(*pagC*-STM14_1502)::Cam	48
JM110	*rpsL thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*)	Stratagene
YD039	14028 Δ(*avrA-invH*)*1*	56
Plasmids		
pASD5006	pWSK29 *fraR-fraBDAE* (Amp^r)	48
pWSK29	pSC101 cloning vector (Amp^r)	48

model is the CBA/J mouse. These mice are *Nramp1*$^{+/+}$ and are resistant to systemic *Salmonella* infection. However, these mice have the unusual attribute of allowing persistent intestinal colonization by *Salmonella*. It was recently discovered that the gastrointestinal tracts of these mice are becoming inflamed during these persistent infections, starting at 10 days postinfection (27, 38). Since this inflammation requires no streptomycin-mediated disruption of the microbiota, it has the most realistic microbial community composition of the *Salmonella* inflammation models.

In this report, we tested a probiotic approach to the prevention and treatment of salmonellosis. Probiotic microbial strains have long been used to prevent or treat illness. Probiotics could potentially replace antibiotics as growth promoters in agriculture or for prophylactic or therapeutic use in humans and animals. More research is needed to identify or design probiotic bacteria and to determine their mechanisms of action (39). With regard to specific infections, a collection of 11 *Lactobacillus* strains or a single *Bacillus* isolate has been found to be effective at reducing *Salmonella* colonization of poultry (40, 41). A probiotic *Escherichia coli* strain isolated from a healthy soldier in World War I, now called *E. coli* Nissle 1917 (here referred to as Nissle), has been shown to reduce *Salmonella* infection in a mouse model (42). This strain is closely related to uropathogenic isolates of *E. coli* but lacks virulence factors and has an abundance of fitness determinants, including at least six iron acquisition systems (43). Competition for iron is one mechanism by which Nissle inhibits *Salmonella* (42). Nissle is safe for use in animals and humans and has been shown to be effective in treating diarrhea, ulcerative colitis, and constipation and preventing necrotizing enterocolitis (NEC) in infants (44–47).

Here, we attempt to enhance the ability of Nissle to compete with *Salmonella* by adding the *Salmonella fra* locus to the Nissle genome. The *fra* locus contains five genes that confer the ability to utilize fructose-asparagine (F-Asn) as a carbon and nitrogen source (48). Mutants lacking this locus were identified in a genetic screen as highly attenuated in mouse models of inflammation (48). The *fra* locus is widely distributed among the gastrointestinal serovars of *Salmonella* but, like many loci involved with anaerobic metabolism, appears to be undergoing genome degradation in the extraintestinal serovars (2). *Salmonella* encodes two type 3 secretion systems (T3SS) encoded within *Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2, respectively) that inject more than 40 effector proteins into host cells (10, 49–52). SPI1 elicits invasion of host cells, while SPI2 is required for survival within host cells. Loss of both renders *Salmonella* unable to cause inflammation and enterocolitis (33, 53). Consistent with a role in enterocolitis, the *fra* locus conferred a fitness benefit upon *Salmonella* only in mouse models that become inflamed from *Salmonella* infection and failed to confer a benefit in strain backgrounds lacking the ability to cause inflammation (lacking SPI1 and SPI2) (48). Therefore, we hypothesized that F-Asn is a significant nutrient source for *Salmonella* during inflammation and that adding the *fra* locus to Nissle would allow Nissle to compete with *Salmonella* for F-Asn and prevent or treat disease. Assuming that adding the *fra* locus to Nissle was going to increase the effectiveness of Nissle, we also pondered adding more *Salmonella*-specific nutrient acquisition systems to Nissle to increase effectiveness further. However, we realized that as we added these loci to Nissle, we would in effect be creating an avirulent *Salmonella* strain. To determine the effectiveness of this strategy, we included a *Salmonella* mutant lacking SPI1 and SPI2 as an example of an avirulent *Salmonella* strain that retains all of its nutrient acquisition loci.

MATERIALS AND METHODS

Strains and media. Bacterial strains are listed in Table 1. Bacteria were grown in LB broth or on LB agar plates for routine culture (EM Science). XLD agar was used for recovery of *Salmonella* from mice (Becton, Dickinson). M9 minimal medium was made as described previously and contained either 5 mM glucose or 5 mM fructose-asparagine (F-Asn) as a carbon source (54). F-Asn was synthesized as previously described (48). When necessary, ampicillin (Amp) or kanamycin (Kan) was added to medium at 200 mg/liter or 60 mg/liter, respectively.

Addition of the *Salmonella fra* locus to *E. coli* Nissle 1917. The low-copy-number plasmid pASD5006, containing the *fra* locus of *Salmonella* strain 14028, or the vector pWSK29 was electroporated into the *E. coli dam dcm* strain JM110 to decrease methylation and then purified and electroporated into *E. coli* Nissle 1917 with selection on LB-Amp. The

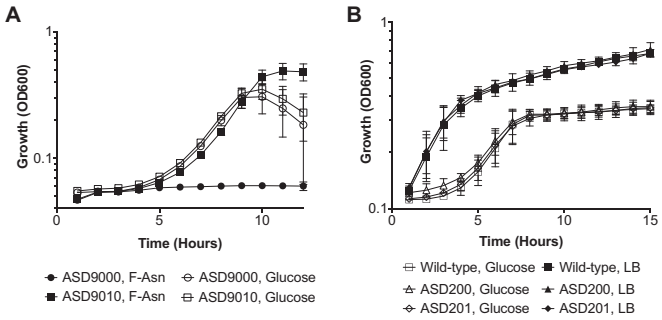

FIG 1 (A) Growth of Nissle plus vector (ASD9000) or Nissle plus *fra* (ASD9010) in M9 minimal medium containing either 5 mM glucose or 5 mM F-Asn as carbon source. (B) Growth of wild-type *Salmonella* (14028), the SPI1 SPI2 mutant (ASD200), and the SPI1 SPI2 *fra* mutant (ASD201) in either M9 glucose or LB.

ability of Nissle to grow on F-Asn was confirmed by growing Nissle plus pASD5006 (ASD9010) in M9 minimal medium with F-Asn as the sole carbon source compared to Nissle plus pWSK29 (ASD9000) (Fig. 1). This was done in a 96-well clear-bottom plate with the optical density at 600 nm recorded over an 18-h period at 37°C using a SpectraMax M5 microplate reader (Molecular Devices) and SoftMax Pro 6.1 software.

Construction of a *Salmonella* SPI1 SPI2 mutant. Lambda Red mutagenesis was used to construct the SPI2 mutant ASD100 (55). Oligonucleotides, including 40 nucleotides matching either *ssrB* or *ssaU*, including 30 nucleotides of the coding region of either target, were appended to sequences that bind pKD4, creating primers BA2558 and BA2559 (55). Oligonucleotides are listed in Table 2. These were used to amplify the Kan cassette from pKD4 using *Taq* DNA polymerase (NEB). The resulting PCR product, an FLP recombination target (FRT)-Kan-FRT cassette flanked by homology to *ssrB* and *ssaU*, was electroporated into strain 14028+pKD46, and transformants were selected on LB-Kan at 37°C. The insertion was verified by PCR using primers BA2582 and BA1922 (K1). This ΔSPI2::Kan mutation was transduced from ASD100 into the ΔSPI1 strain YD039 (56) using phage P22HTint, creating ASD199. The antibiotic resistance marker was deleted by electroporating ASD199 with pCP20 (55), which encodes the FLP recombinase, and transformants were selected on LB-Amp at 30°C. Deletion of the Kan cassette was verified using PCR with primers BA2582 and BA2583, as well as screened for loss of pCP20, creating ASD200.

Construction of SPI1 SPI2 *fra* triple mutant. Lambda Red mutagenesis was used to create an *fraRBDAE* island mutant (STM14_4332-STM14_4328), CS1005, using the protocol described above. Briefly, oligonucleotides BA2515 and BA2538 were used to amplify the Kan cassette from pKD4 using *Taq* DNA polymerase (NEB). The PCR product was electroporated into 14028+pKD46, and transformants were selected on LB-Kan at 37°C to create CS1005. The insertion of the Kan cassette was verified by PCR using BA1922 (K1) and BA2888. The resulting (*fraR-fraBDAE*)4::Kan island mutation was transduced into the ΔSPI1 ΔSPI2 strain ASD200 using the phage P22HTint, creating ASD201.

Animals. Swiss Webster mice were obtained from Taconic Farms. CBA/J mice were obtained from Jackson Laboratories. Germfree C57BL/6 and Swiss Webster mice were bred at the Ohio State University (OSU) germfree animal facility. All mice were females between 6 and 10 weeks of age. All bacterial inocula were grown with shaking at 37°C overnight, resuspended in water, and administered by the intragastric route in a volume of 200 μl. For survival curves, mice were euthanized upon reaching the early-removal criteria of our approved animal protocol. For CFU determinations, ceca or feces were homogenized in 3 ml or 0.75 ml, respectively, of phosphate-buffered saline (PBS). One-hundred-microliter aliquots of serial dilutions were then plated on XLD agar plates containing the appropriate antibiotics, yielding a detection limit of 30 CFU for ceca and 8 CFU for feces.

Histopathology. Cecal samples were removed from mice, and a portion was immersion fixed in 10% neutral buffered formalin, processed by routine methods, and embedded in paraffin wax by the Comparative Pathology and Mouse Phenotyping Shared Resource at the Ohio State University. Sections (4 μm) were stained with hematoxylin and eosin (H&E) and scored in a blinded fashion by a veterinary pathologist, board certified by the American College of Veterinary Pathologists (ACVP). The adapted, semiquantitative histopathologic scoring system (57) assessed enterocyte loss (none, 0; loss of single cell, 1; loss of groups of cells/erosion, 2; overt ulceration, 3), crypt inflammation (none, 0; 1 to 2 inflammatory cells, 1; cryptitis, 2; crypt abscess, 3), mononuclear cell inflammation (none, 0; mild, 1; moderate, 2; marked, 3), neutrophilic inflammation (none, 0; mild, 1; moderate, 2; marked, 3), epithelial hyperplasia (none, 0; mild, 1; moderate, 2; discrete nests of regenerated crypts delineated from adjacent

TABLE 2 Oligonucleotides

Oligonucleotide	Sequence	Description
BA1922	CAGTCATAGCCGAATAGCCT	Kanamycin cassette insertion verification primer
BA2515	GCCTGCATGATTAATACGTACTGAAATAACTCTGGATCAGCATATGAATATCCTCCTTAG	Lambda Red mutagenic reverse primer for STM14_4328 with P2 priming site
BA2538	ATGGATACAAATGATCGAGCAACCCGACAGTAAAAGCGCCGTGTAGGCTGGAGCTGCTTC	Lambda Red mutagenic forward primer for STM14_4332 with P1 priming site
BA2558	ACGCCCCTGGTTAATACTCTATTAACCTCATTCTTCGGGCGTGTAGGCTGGAGCTGCTTC	Lambda Red mutagenic forward primer with homology to *ssrB* with P1 priming site
BA2559	CCAAAAGCATTTATGGTGTTTCGGTAGAATGCGCATAATCCATATGAATATCCTCCTTAG	Lambda Red mutagenic reverse primer with homology to *ssaU* with P2 priming site
BA2582	AAATAAGGGGATTCTACTATATCATGATCA	Reverse primer for confirmation of SPI2 deletion
BA2583	GCCAGGCTAAAAGCGATTATTTTCAGTCTC	Forward primer for confirmation of SPI2 deletion
B2888	GGATCCGCTTCGATACCTGAGTGGCAAAGTGTGCG	Forward primer for verification of *fra* island mutation with K1

mucosa with no obvious disruption from overlying mucosal surface, 3), and edema (none, 0; mild/focal/single layer affected, 1; moderate/multifocal/multiple layers affected, 2; marked/widespread/transmural involvement, 3).

Animal assurance. All animal work was performed in accordance with the protocols approved by our Institutional Animal Care and Use Committee (OSU 2009A0035). The IACUC ensures compliance of this protocol with the U.S. Animal Welfare Act, the *Guide for the Care and Use of Laboratory Animals* (63), and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

RESULTS

A *fra* mutant of *Salmonella* is attenuated in several murine inflammation models, suggesting that F-Asn is a nutrient that is important to *Salmonella* fitness in the inflamed intestine (48). Therefore, we hypothesized that adding the *fra* locus to a probiotic organism would enhance the ability of that organism to compete with *Salmonella* for F-Asn and prevent or treat *Salmonella* infections. To test this hypothesis, we cloned the *Salmonella fra* locus on a low-copy-number plasmid and placed this plasmid in the well-characterized probiotic strain *E. coli* Nissle 1917 (Nissle). As expected, Nissle carrying the *fra* plasmid (ASD9010) was able to grow on F-Asn as the sole carbon source while Nissle carrying the vector alone (ASD9000) was not (Fig. 1A). We considered adding more *Salmonella*-specific nutrient acquisition systems to Nissle but realized that this was much like creating a nonpathogenic *Salmonella* strain. Therefore, instead of adding more nutrient acquisition systems to Nissle, we constructed a mutant of *Salmonella* lacking SPI1 and SPI2 (ASD200). This strain should compete with wild-type *Salmonella* for all nutrients without causing disease. In later experiments, we also constructed and tested an SPI1 SPI2 *fra* triple mutant (ASD201) to determine the *fra* dependence of any observed effects. Both ASD200 and ASD201 grow similarly to the wild type in M9 glucose and LB (Fig. 1B). We will refer to these four strains as the "probiotics" throughout this report.

To determine if the probiotics could protect mice from wild-type *Salmonella*, we started with germfree mice, which have no colonization resistance. We used both Swiss Webster and C57BL/6 mice (*Nramp1*$^{+/+}$ and *Nramp1*$^{G169D/G169D}$, respectively). A 10^9-CFU quantity of a probiotic strain or sham (water) was administered by oral gavage to groups of five mice. The following day, the mice were challenged with a lethal dose of 10^4 CFU of virulent *Salmonella* (strain JLD1214, which is a chloramphenicol-resistant derivative of ATCC 14028). In both germfree C57BL/6 mice and germfree Swiss Webster mice, all of the probiotics enhanced survival compared to sham (Fig. 2). Nissle plus *fra* protected slightly better than Nissle plus vector in germfree C57BL/6 mice, but this was not statistically significant ($P = 0.075$). Interestingly, Nissle plus vector was highly protective in germfree Swiss Webster mice (100% survival), but Nissle plus *fra* was less protective, with no survival ($P = 0.004$). The *Salmonella* SPI1 SPI2 mutant was the most protective in both types of mice. The *Salmonella* triple mutant (SPI1 SPI2 *fra*) was used only in the germfree Swiss Webster mice. While it appeared less protective than the double mutant (SPI1 SPI2), this was not statistically significant ($P = 0.091$).

To test the safety of the probiotics, each strain was administered at a dose of 10^9 CFU to a group of germfree mice and mortality was monitored (Fig. 3). The *Salmonella* SPI1 SPI2 mutant and Nissle plus vector were completely safe in both types of mice (no mortality). Nissle plus *fra* caused no mortality in the Swiss Webster mice but caused 100% mortality in the C57BL/6 mice (Fig. 3). This indicates that the addition of the *fra* locus to Nissle increased its virulence in germfree C57BL/6 mice. In a separate experiment, we infected germfree Swiss Webster mice with a dose of 10^9 CFU of the *Salmonella* SPI1 SPI2 mutant and then quantitated inflammation of the ceca after 6 days of colonization using histopathology. The *Salmonella* SPI1 SPI2 mutant was safe with regard to inflammation (Fig. 3).

The experiments in germfree mice revealed that the Nissle-plus-*fra* strain was less effective than Nissle plus vector at preventing death in germfree Swiss Webster mice (Fig. 2B), and it gained the ability to kill germfree C57BL/6 mice (Fig. 3A). Thus, the ability to utilize F-Asn enhanced the virulence of Nissle. In contrast, the *Salmonella* SPI1 SPI2 mutant was safe and effective in protecting both germfree C57BL/6 and germfree Swiss Webster mice from wild-type *Salmonella*.

To further test the ability of these strains to protect against a lethal *Salmonella* infection, we moved to a Strep-treated Swiss Webster mouse model. Mice with a normal microbiota are highly resistant to *Salmonella*-mediated inflammation, but treatment with streptomycin disrupts the microbiota and allows *Salmonella*-mediated inflammation to occur within a day of infection. Thus, in this experiment the mice were treated with streptomycin. One day later, they were treated with a dose of 10^9 CFU of a probiotic strain or sham; 1 day after that, they were challenged with a lethal dose of *Salmonella* (10^7 CFU of JLD1214). All of the probiotic strains except Nissle plus vector provided statistically significant protection compared to sham (Fig. 4). The protection provided by Nissle plus *fra* was statistically significantly different from that of sham but was not different from that of Nissle plus vector ($P = 0.523$), making it difficult to conclude that the ability to utilize F-Asn improved the ability of Nissle to protect against *Salmonella* (Fig. 4). The *Salmonella* SPI1 SPI2 mutant and the SPI1 SPI2 *fra* triple mutant both provided protection statistically significantly different from that provided by sham, but they were not different from each other ($P = 0.684$), indicating that protection is not dependent upon the ability to utilize F-Asn (Fig. 4).

A more recent mouse model of *Salmonella*-mediated inflammation is the CBA/J model. These mice are *Nramp1*$^{+/+}$, they tend to carry *Salmonella* for long periods in their intestinal tract, and their intestinal tract becomes inflamed by day 10 postinfection (27, 38). With no need for disruption of the microbiota with antibiotics, this model may have the most realistic microbiota composition during inflammation. To test the ability of our probiotic strains to treat a *Salmonella* infection, we inoculated the CBA/J mice with 10^9 CFU of *Salmonella*, waited 10 days for inflammation to begin, and then treated the mice with 10^9 CFU of probiotic or sham. Thus, this is a therapeutic rather than a prophylactic model. *Salmonella* shedding in feces was measured on days 10 (just before probiotic inoculation), 11, and 13, and ceca were harvested on day 17 (Fig. 5). Nissle plus *fra* appeared to reduce *Salmonella* shedding in ceca by day 17, but this just missed statistical significance, with a *P* value of 0.055. The only probiotic strain to cause a statistically significant decrease in fecal counts of virulent *Salmonella* over time was the *Salmonella* SPI1 SPI2 mutant (day 13 compared to day 10). The SPI1 SPI2 *fra* triple mutant was not different over time, which might suggest that protection is *fra* dependent; however, it was not different from the SPI1 SPI2 mutant either ($P = 0.999$), leaving the *fra* dependence unlikely.

We used the CBA/J model a second time, in which we increased

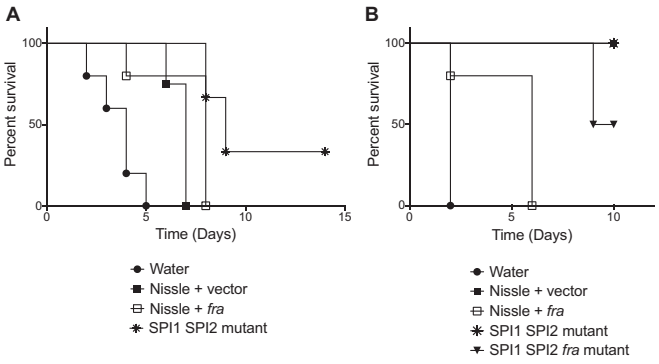

FIG 2 Evaluation of probiotics as prophylactics in germfree mice. On consecutive days, groups of five germfree C57BL/6 mice (A) or germfree Swiss Webster mice (B) were orally administered a probiotic strain (10^9 CFU of strains listed below the graph) or sham (water) and then virulent *Salmonella* (10^4 CFU of JLD1214). Survival was monitored over time. The statistical significance of each treatment being different from the others was determined with log rank (Mantel-Cox) tests, without correction for multiple comparisons, with a *P* value of <0.05 considered significant. In both panels, the sham was statistically significantly different from all of the treatments ($P < 0.014$ or better). In panel A, Nissle plus vector was not different from Nissle plus *fra* ($P = 0.07$), but it was different from the SPI1 SPI2 mutant ($P = 0.02$). In panel B, Nissle plus vector was different from Nissle plus *fra* ($P = 0.003$) but was not different from the SPI1 SPI2 mutant ($P = 1.0$) or the SPI1 SPI2 *fra* mutant ($P = 0.09$). In panel B, the SPI1 SPI2 mutant was not different from the SPI1 SPI2 *fra* mutant ($P = 0.09$).

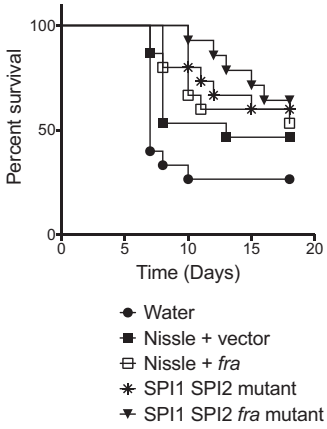

FIG 4 Evaluation of probiotics as prophylactics in Strep-treated Swiss Webster mice. On consecutive days, groups of 15 mice were administered streptomycin, then a probiotic strain (10^9 CFU of strains listed below the graph) or sham (water), and then virulent *Salmonella* (10^7 CFU of JLD1214). Survival was monitored over time. The statistical significance of each treatment being different from the others was determined with log rank (Mantel-Cox) tests, without correction for multiple comparisons, with a *P* value of <0.05 considered significant. The sham is statistically significantly different from all treatments ($P < 0.03$ or better) except Nissle plus vector ($P = 0.10$). Nissle plus vector is not different from Nissle plus *fra* ($P = 0.52$), SPI1 SPI2 mutant ($P = 0.27$), or the SPI1 SPI2 *fra* mutant ($P = 0.40$). The SPI1 SPI2 mutant is not different from the SPI1 SPI2 *fra* mutant ($P = 0.68$).

the number of mice per group from 5 to 8 and increased the number of probiotic doses from one to three, administered on days 10, 12, and 14 postinfection (Fig. 6). As in the previous experiment, only the SPI1 SPI2 mutant reduced the counts of virulent *Salmonella* over time (day 14 compared to day 10). Again, the SPI1 SPI2 *fra* triple mutant was not different over time, suggesting that there is *fra* dependence to the protection. However, the triple mutant was not different from the double mutant ($P = 0.527$), again leaving the *fra* dependence in question. For this experiment, we also performed histopathology on ceca harvested on day 15 to determine if inflammation was reduced by the probiotics. This showed that there were no statistically significant differences between the treatment and sham groups (Fig. 7). However, the mice treated with the *Salmonella* SPI1 SPI2 mutant appeared to fall into two categories, with half having little or no inflammation while the other half were highly inflamed. As a group, there may be no statistically significant improvement, but for some individuals, the treatment may be effective. Consistent with this, the only mice that were completely cleared of wild-type *Salmonella* from their cecum were two mice that had been treated with the *Salmonella* SPI1 SPI2 mutant and one mouse that had been treated

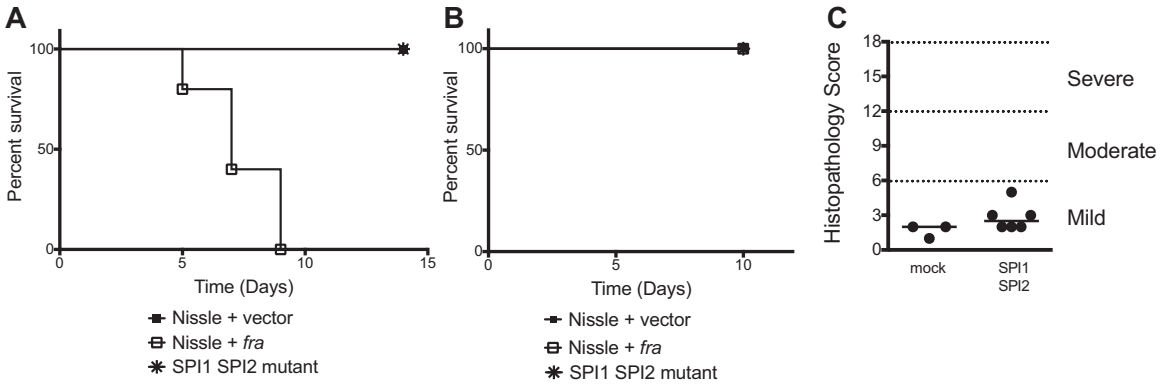

FIG 3 (A and B) Safety of probiotics in germfree C57BL/6 mice (A) and germfree Swiss Webster mice (B). Groups of five mice were orally administered a probiotic strain (10^9 CFU of strains listed in the legend), and survival was monitored over time. (C) A group of six germfree Swiss Webster mice were orally administered 10^9 CFU of the *Salmonella* SPI1 SPI2 mutant, while three mice were inoculated with water (mock). After 6 days, the mice were euthanized and their ceca were scored for inflammation using histopathology. Bars indicate the median. In panels A and B, the statistical significance of each treatment being different from the others was determined with log rank (Mantel-Cox) tests, without correction for multiple comparisons, with a *P* value of <0.05 considered significant. In panel A, the Nissle plus *fra* was significantly different from Nissle plus vector and SPI1 SPI2 mutant ($P = 0.0026$). In panel B, the groups were not different. In panel C, the statistical significance of differences between groups was determined using a Mann-Whitney test and the groups were not different ($P = 0.13$).

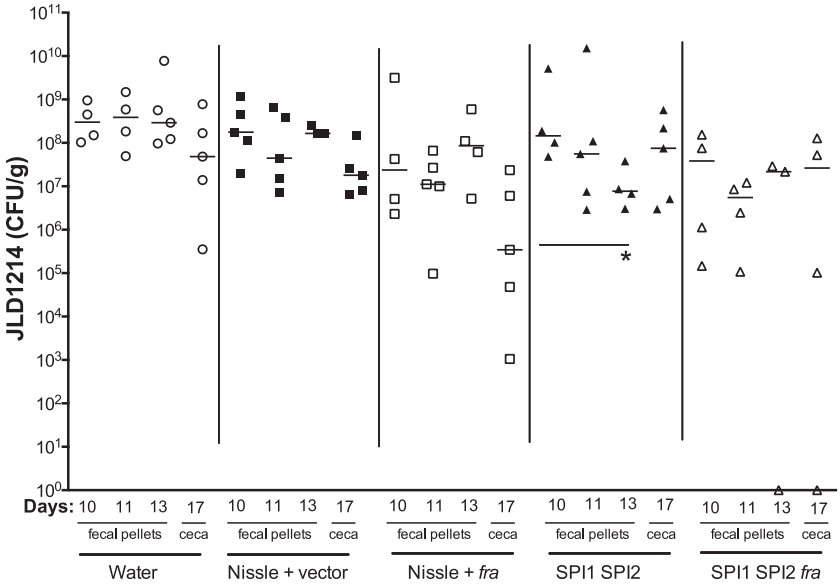

FIG 5 CBA/J mice were orally inoculated with 10^9 CFU of virulent *Salmonella* strain JLD1214. Ten days postinfection, groups of five mice were treated with 10^9 CFU of probiotic or sham. *Salmonella* (JLD1214) shedding in feces was measured on days 10 (just before probiotic inoculation), 11, and 13. *Salmonella* (JLD1214) in the ceca was measured on day 17. The limit of detection was 30 CFU for ceca and 8 CFU for feces. Statistical significance between groups was determined using a Mann-Whitney test. *, $P < 0.05$.

with the *Salmonella* SPI1 SPI2 *fra* triple mutant (Fig. 6). These three mice also had the lowest inflammation scores in their respective groups.

The CBA/J model demonstrated that the *Salmonella* SPI1 SPI2 mutant can reduce the CFU counts of wild-type *Salmonella* in fecal samples, but this was not a dramatic effect. This may be because the wild type had a 10-day head start before the probiotic was administered. We decided to return to the Strep-treated Swiss Webster model, but rather than testing the ability of the *Salmonella* SPI1 SPI2 mutant to prevent an infection, as in Fig. 4, we would use the mutant to treat an existing infection. In this experiment, the mice were treated with streptomycin; the following day, they received either 10^7, 10^8, or 10^9 CFU of wild-type *Salmonella* (JLD1214). Then, 24 h later, they received 10^9 CFU of probiotic or sham (water). Survival was monitored over time (Fig. 8). At 10^7 and 10^8 CFU of wild-type *Salmonella*, administration of the SPI1 SPI2 mutant had no effect on the survival curve (Fig. 8A and B). At 10^9 CFU of wild-type *Salmonella*, administration of the SPI1 SPI2 mutant appeared to improve survival of the mice, but this was not statistically significant (Fig. 8C).

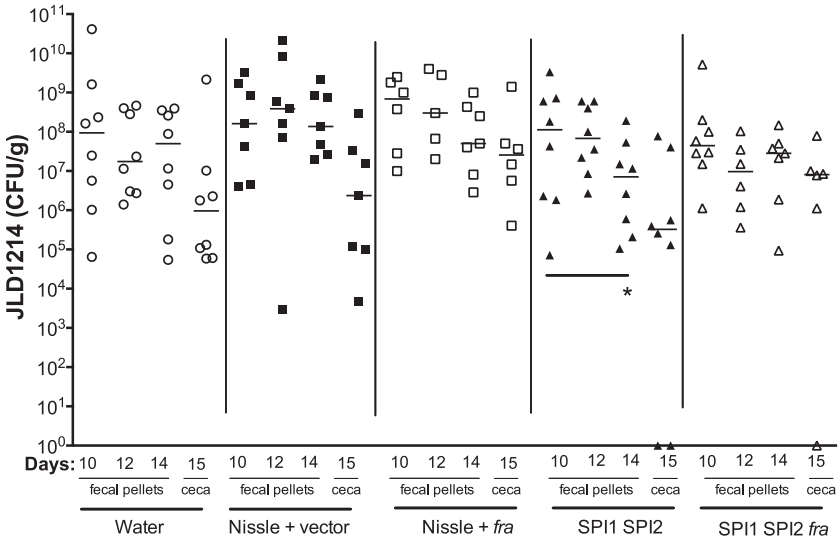

FIG 6 CBA/J mice were orally inoculated with 10^9 CFU of virulent *Salmonella* strain JLD1214. Groups of eight mice were treated with 10^9 CFU of probiotic or sham three times, on days 10, 12, and 14 postinfection. *Salmonella* (JLD1214) shedding in feces was measured on the same days just before probiotic inoculation. *Salmonella* (JLD1214) in the ceca was measured on day 15. The limit of detection was 30 CFU for ceca and 8 CFU for feces. Statistical significance between groups was determined using a Mann-Whitney test. *, $P < 0.05$.

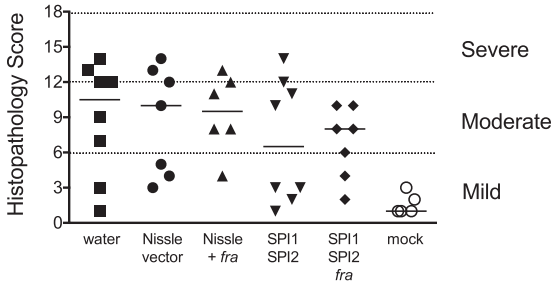

FIG 7 Histopathology scores of ceca harvested from the mice in Fig. 6 on day 15 postinfection. The bar represents the median.

DISCUSSION

The *fra* locus was identified in a genetic screen for *Salmonella* genes that are differentially required for fitness in germfree mice colonized, or not, with the commensal organism *Enterobacter cloacae* (48). Further experimentation revealed that a *fraB* mutation was severely attenuated in its ability to compete with wild-type *Salmonella* in four mouse models of inflammation: germfree, germfree colonized with human fecal microbiota, Strep treated, and interleukin-10 (IL-10) knockout. Interestingly, the *fraB* mutant was not attenuated in conventional mice that fail to become inflamed from *Salmonella* infection. It was also determined that a *fraB* mutant has no phenotype if the competition experiment is performed in a *Salmonella* genetic background lacking SPI1 and SPI2. These results were interpreted to mean that SPI1 and SPI2 are required for *Salmonella* to induce inflammation (in models that are permissive), and the inflammation may be killing microbes that would otherwise compete for F-Asn (48). This model gave rise to the idea that adding the *fra* locus to probiotic species, such as *E. coli* Nissle 1917, could give them the ability to compete with *Salmonella* for a critical nutrient source and thus prevent infection. However, since then we have learned that the *fraB* phenotype is primarily due to the accumulation of a toxic metabolite during growth on F-Asn rather than F-Asn being a critical nutrient source (B. M. M. Ahmer, unpublished data). Despite this, there seemed to be some *fra* dependence with regard to the ability of the *Salmonella* SPI1 SPI2 mutant to compete with wild-type *Salmonella*, especially in CBA/J mice. It appeared that protection was *fra* dependent because the SPI1 SPI2 double mutant, but not the SPI1 SPI2 *fra* triple mutant, was significantly different from sham. However, the double mutant is not statistically significantly different from the triple mutant, leaving the *fra* dependence in question. Furthermore, the Nissle strain modified to contain the *fra* locus was altered in its ability to kill germfree C57BL/6 mice and in its ability to protect germfree Swiss Webster mice against *Salmonella* infection, compared to the original Nissle strain. These results suggest that F-Asn is a significant nutrient source in some situations but definitely not the only nutrient source available to *E. coli* and *Salmonella* in the inflamed intestine.

The mechanism by which virulence of Nissle is enhanced by the *fra* locus is unknown. Virulence enhancement was observed only in germfree mice and resulted in the killing of C57BL/6 mice and a reduced ability to protect Swiss Webster mice against *Salmonella*, compared to wild-type Nissle (Nissle plus vector). We have unpublished results that indicate that F-Asn concentrations are quite high in the intestines of germfree mice. However, it is still surprising that simply providing another nutrient source to Nissle had these effects. The C57BL/6 mice are mutated at the *Nramp1* locus, while the Swiss Webster mice are not, which makes the C57BL/6 mice more susceptible to systemic infections. It is possible that Nissle carrying the *fra* locus simply grew to higher numbers in the intestine, which allowed escape to a permissive systemic environment. In the Swiss Webster mice, wild-type Nissle was 100% effective in preventing killing of the mice by *Salmonella*, while Nissle plus *fra* delayed the killing compared to sham but still resulted in no survival. Why Nissle plus *fra* would have a reduced ability to protect against *Salmonella* is unclear. While adding Nissle to *fra* was not a successful strategy, this does not rule out the possibility that adding *fra* to a different probiotic organism, such as *Lactobacillus* or *Bifidobacterium*, might enhance the ability of these organisms to compete with *Salmonella*.

The *Salmonella* SPI1 SPI2 mutant looks promising. This strain was included in the study to determine what would happen if we continued adding *Salmonella*-specific nutrient acquisition loci to Nissle, essentially creating an avirulent *Salmonella* strain. Unlike Nissle, the *Salmonella* SPI1 SPI2 mutant has all of the same nutrient acquisition loci as does wild-type *Salmonella*. This strain was safe, and noninflammatory, even at doses of 10^9 CFU in highly susceptible germfree C57BL/6 mice (Fig. 3). This strain was also effective at prevention of *Salmonella* infection using the germfree

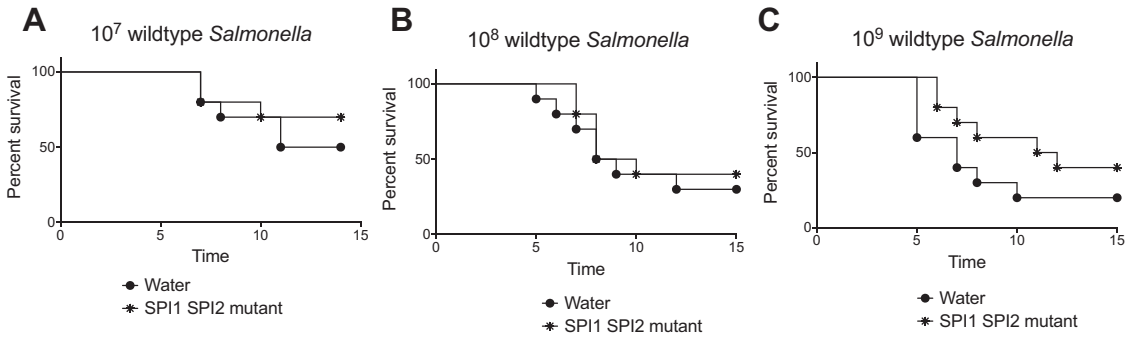

FIG 8 Evaluation of the *Salmonella* SPI1 SPI2 mutant as a therapeutic in the Strep-treated Swiss Webster model. On consecutive days, groups of 10 mice were administered streptomycin, then wild-type *Salmonella* JLD1214 (10^7 CFU in panel A, 10^8 CFU in panel B, or 10^9 CFU in panel C), and then 10^9 CFU of the probiotic (*Salmonella* SPI1 SPI2 mutant, ASD200) or sham (water). Survival was monitored over time. The statistical significance of each treatment being different from the others was determined with log rank (Mantel-Cox) tests, without correction for multiple comparisons, with a P value of <0.05 considered significant. The sham was not statistically significantly different from the treatment in panel A ($P = 0.44$), panel B ($P = 0.58$), or panel C ($P = 0.17$).

and Strep-treated models (Fig. 2 and 4). A much more difficult task is to treat an existing infection. The *Salmonella* SPI1 SPI2 mutant was indeed modestly effective at treating an existing infection using the CBA/J model (Fig. 5 and 6) but not the Strep-treated Swiss Webster model (Fig. 8). In the CBA/J model, the wild-type *Salmonella* was administered 10 days before the probiotic. Despite being administered 10 days after the wild-type *Salmonella*, the probiotic was able to reproducibly reduce the CFU of wild-type *Salmonella*. Overall, however, the *Salmonella* SPI1 SPI2 mutant is much more effective as a preventative than as a therapeutic.

Currently, a *cya crp* mutant of *Salmonella* is used as a live attenuated vaccine strain in agriculture (58–60). This strain is metabolically attenuated so that it cannot compete metabolically with wild-type *Salmonella* but instead creates a lasting immune response against a single serovar. The use of a *Salmonella* SPI1 SPI2 mutant as a probiotic takes a different approach in which the strain is metabolically competent, so that it may be able to compete effectively against hundreds of serovars of *Salmonella*. There is precedent for this approach in the literature. A nontoxigenic *Clostridium difficile* strain can compete with wild-type *C. difficile* to resolve infection and prevent recurrence (61). Of the different mouse models, the *Salmonella* SPI1 SPI2 mutant was the most effective in protecting germfree mice from wild-type *Salmonella*. This suggests that this strain might be particularly effective in preventing *Salmonella* colonization of neonatal agricultural animals such as newly hatched poultry or swine. The probiotic approach could be used as an alternative, or in conjunction with vaccination, as the probiotic may protect during the first week or two of life while responses to vaccination are developing.

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