

REPORT

MICROBIOTA

Neonatal acquisition of *Clostridia* species protects against colonization by bacterial pathogens

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The high susceptibility of neonates to infections has been assumed to be due to immaturity of the immune system, but the mechanism remains unclear. By colonizing adult germ-free mice with the cecal contents of neonatal and adult mice, we show that the neonatal microbiota is unable to prevent colonization by two bacterial pathogens that cause mortality in neonates. The lack of colonization resistance occurred when Clostridiales were absent in the neonatal microbiota. Administration of Clostridiales, but not Bacteroidales, protected neonatal mice from pathogen infection and abrogated intestinal pathology upon pathogen challenge. Depletion of Clostridiales also abolished colonization resistance in adult mice. The neonatal bacteria enhanced the ability of protective Clostridiales to colonize the gut.

Newborns and children less than 1 year old are highly susceptible to frequent infection by orally acquired bacterial pathogens (1, 2). Susceptibility to intestinal infections in neonates has been generally ascribed to immaturity of the innate and adaptive immune systems; however, additional factors may play a role because immune responses to different stimuli are highly variable among neonates (3). The gut microbiota is important to the development of the immune system (4, 5). For example, gut microbiota-induced local responses, such as secretory immunoglobulin A, as well as local T helper 17 cells and regulatory T cells, contribute to gut homeostasis. Another major attribute of the microbiota is to protect the host against colonization by exogenous pathogens, a function

termed “colonization resistance” (4, 6). The gut microbiota of neonates is less diverse than that of adult individuals and tends to lack Clostridiales and Bacteroidales, the dominant taxa found in the adult intestine (7, 8).

To compare the function of the neonatal and the adult microbiota in colonization resistance against pathogens independently of the age of the host, we colonized age-matched adult germ-free (GF) mice with the cecal contents of neonatal mice or adult (7-week-old) mice and kept the reconstituted mice in isolators to prevent contamination with exogenous bacteria. Analysis of the 16S ribosomal RNA (rRNA) gene of the fecal microbiota 21 days after reconstitution revealed that the bacterial composition of adult GF mice colonized with the microbiota from 4-day-old mice resembled that of the donor and was dominated by facultative anaerobes including Lactobacillaceae but devoid of Clostridiales and Bacteroidales (Fig. 1A and fig. S1A). The microbiota of GF mice reconstituted with feces from 12-day-old mice was dominated by operational taxonomic units (OTUs) belonging to the Enterobacteriaceae and Lactobacillaceae families, and few OTUs belonging to the Lachnospiraceae family compared with that of 16-day-old and adult mice (Fig. 1A and fig. S1, A and B). In contrast, strict anaerobic bacteria with a large number of Clostridiales OTUs belonging to Lachnospiraceae and Ruminococcaceae families, as well as Porphyromonadaceae and unclassified Bacteroidales, were prevalent in GF mice colonized with the cecal contents of 16-day-old or adult mice (Fig. 1A and fig. S1, A and B).

Consistently, there was a greater diversity in the microbiota of GF mice reconstituted with 16-day-old and adult mice than in mice colonized with the cecal contents of 4-day-old and 12-day-old mice (Fig. 1B). To assess the ability of the different microbiotas to control pathogen replication in the intestine in the absence of systemic invasion, we intragastrically infected reconstituted GF mice with a *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) mutant deficient in the type III secretion system (T3SS) encoded by *Salmonella* pathogenicity island 2 (Δ *spiA*), which replicates normally in the intestine but is deficient in systemic spread (9, 10). We found that ~50% of GF mice colonized with the microbiota of 4-day-old mice succumbed to *S. Typhimurium* infection, whereas all GF mice colonized with the adult microbiota survived (Fig. 1C).

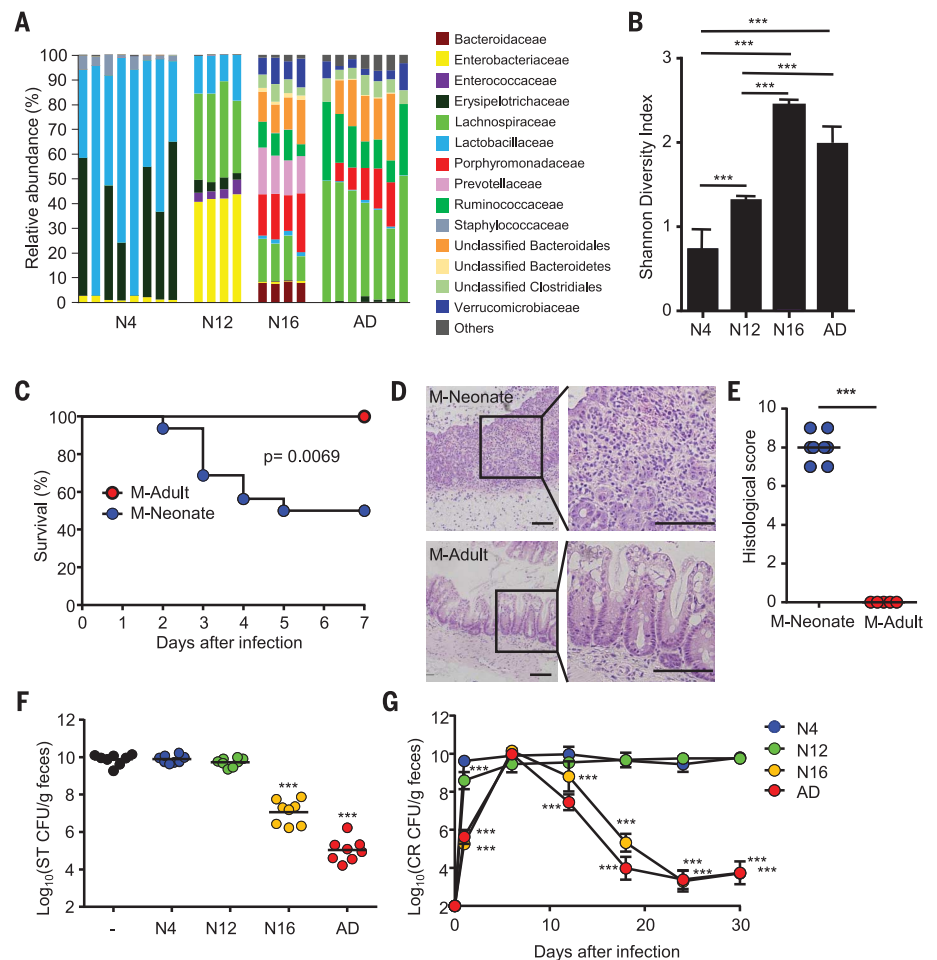
The increased mortality of GF mice harboring a microbiota from 4-day-old mice was associated with marked intestinal cell damage, submucosal edema, and inflammatory cell infiltrates in the cecum, which were absent in GF mice colonized with the microbiota of adult mice (Fig. 1, D and E). Consistent with these findings, ~80% of 7-day-old mice infected with *S. Typhimurium* Δ *spiA* succumbed, whereas all adult mice survived the infection (fig. S2). Notably, GF mice colonized with the microbiota from 4- and 12-day-old mice harbored ~4- to 5-logs-higher pathogen loads in the feces than mice colonized with the microbiota from 16-day-old or adult mice (Fig. 1F). To assess the function of the microbiota in colonization resistance against another enteric pathogen, we intragastrically infected adult GF mice reconstituted with the microbiota of neonatal and adult mice with *Citrobacter rodentium*, a natural pathogen of mice that is used to model human infections with enteropathogenic *Escherichia coli* (11). Consistent with the *S. Typhimurium* results, GF mice colonized with microbiota from 4- and 12-day-old mice were impaired in resisting colonization of *C. rodentium* as shown by 6-logs-higher pathogen loads in the feces by day 25 to 30 after infection, in comparison with mice colonized by the microbiota from 16-day-old or adult mice (Fig. 1G). These results indicate that the gut microbiota from neonatal mice are not able to mediate colonization resistance against *S. Typhimurium* and *C. rodentium*.

We next determined whether the adult microbiota can confer colonization resistance to mice harboring a neonatal microbiota. To assess this, GF mice colonized with the microbiota of 4-day-old mice were orally infected with *C. rodentium*, and on day 30 after infection the adult microbiota was transferred to infected mice by cohousing. Notably, the burden of *C. rodentium* in GF mice harboring the day 4 neonatal microbiota declined by ~5 logs after 5 days and was further reduced by ~6 logs by day 7 of cohousing with adult mice (Fig. 2A). These findings suggest that addition of symbiotic bacteria present in adult mice to the neonatal microbiota is sufficient for colonization resistance. We asked next whether depletion of the microbiota of adult mice to a state comparable with that

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Fig. 1. The early neonatal microbiota lacks colonization resistance against enteric pathogens.

(A) Relative abundance of operational taxonomic units (OTUs) in fecal samples from GF mice reconstituted with cecal contents of 4-day-old (N4) ($n = 8$), 12-day-old (N12) ($n = 4$), 16-day-old (N16) ($n = 4$), or 7-week-old (AD) ($n = 7$) mice. Analysis was performed on day 21 after reconstitution. Colors correspond to families. Data are combined from two independent experiments ($n = 2$ to 4 in each experiment). **(B)** Shannon's diversity index of fecal samples from GF mice reconstituted with indicated microbiota. Pooled data from three independent experiments are shown in Fig. 1A. $***P < 0.001$, Dunnett's multiple comparisons test. Results are means \pm SD. **(C to E)** Age- and gender-matched adult GF mice reconstituted with N4 microbiota (M-Neonate) or AD microbiota (M-Adult) were infected with *S. Typhimurium* Δ spiA. **(C)** Mouse survival over time after infection of M-Neonate ($n = 16$) and M-Adult ($n = 11$) mice. Data are from two pooled experiments with $n = 5$ to 8 mice per group, log-rank test. **(D)** Representative histology of hematoxylin and eosin (HE)-stained cecal sections from infected M-Neonate and M-Adult mice. Cecal tissue was processed 1 day after infection. High-power images of marked area are shown in inset. Scale bars, 100 μ m. **(E)** Histopathological scores of cecal tissue from M-Neonate and M-Adult mice infected with *S. Typhimurium*. Each dot represents an individual mouse (M-Neonate, $n = 8$; M-Adult, $n = 5$). Data are representative of two independent experiments. $***P < 0.001$, Mann-Whitney U test. **(F)** Adult GF mice reconstituted with indicated microbiota were infected with *S. Typhimurium* Δ spiA (ST). Pathogen loads [colony-forming units (CFU)/gram] in feces were determined 1 day after infection by plating. Each dot represents an individual mouse. Data are pooled ($n = 8$) from two independent experiments. $***P < 0.001$ versus GF mice, Dunnett's multiple comparisons test. **(G)** GF mice reconstituted with N4, N12, N16, and AD microbiota were infected with *C. rodentium* (CR). CFU/gram of feces were determined on days 1, 3, 6, 12, 18, 24, and 30 after infection. Results are means \pm SD and representative of three independent experiments; $n = 4$ to 5 per experiment [4-day-old (N4), 12-day-old (N12), 16-day-old (N16), or 7-week-old (AD)]. $***P < 0.001$ versus N4, Dunnett's multiple comparisons test.



of the neonatal mice would affect colonization resistance to pathogens. Accordingly, adult mice were treated with a high dose of vancomycin, which selectively kills anaerobes, clears Clostridiales and Bacteroidales, and increases the abundance of facultative anaerobes, including Enterobacteriaceae and Lactobacillaceae (fig. S3). Consistently, the microbiota of mice treated with vancomycin showed less diversity than that of untreated mice (Fig. 2B). Vancomycin-treated mice showed increased pathogen colonization after infection with *C. rodentium* and harbored ~6-logs-higher pathogen loads in the feces by day 25 to 30 after infection when compared with untreated mice (Fig. 2C). Likewise, treatment of adult mice with vancomycin increased *S. Typhimurium* colonization in fecal and cecal contents by 4 to 5 logs (Fig. 2D). In another approach to alter the composition of the adult microbiota, we fed adult mice a lactose- and cellobiose-rich diet for 6 weeks and assessed the fecal microbiota by 16S rRNA gene analysis. The composition of the microbiota in mice fed a lactose- and cellobiose-rich diet re-

sembled that of 4-day-old and 12-day-old mice in that it was dominated by Erysipelotrichaceae, Enterobacteriaceae, Verrucomicrobiaceae, Lactobacillaceae, and Bifidobacteriaceae and depleted of Clostridiales and Bacteroidales (Fig. 2E). Notably, adult mice fed the lactose- and cellobiose-rich diet harbored increased loads of *S. Typhimurium* in fecal and cecal contents when compared with mice fed a conventional diet (Fig. 2F).

The impaired colonization resistance of the neonatal microbiota against enteric pathogens is associated with the absence of or reduced numbers of Clostridiales and Bacteroidales compared with the microbiota of 16-day-old or adult mice. To determine whether Clostridiales or Bacteroidales species are important in mediating colonization resistance, GF mice harboring the microbiota from 4-day-old mice were gavaged with a consortium of mouse *Clostridia* species [(12) and table S1], with a mixture of four mouse *Bacteroides* species, or were left untreated. Notably, administration of *Clostridia*, but not *Bacteroides*, species restored colonization resistance in the

neonatal microbiota against *S. Typhimurium* as determined by analysis of pathogen loads in fecal or cecal contents (Fig. 3A). Administration of a second independent consortium of intestinal Clostridiales also restored colonization resistance, whereas that of a more complex mixture of 12 mouse strains belonging to the order Bacteroidales did not, despite efficient colonization after gavage (fig. S4 and tables S1 and S2). The reduced pathogen loads observed after Clostridiales administration were associated with an increase in the length of the cecum and colon, indicating diminished intestinal inflammation, when compared with untreated mice or mice gavaged with *Bacteroides* (fig. S4). Consistently, *S. Typhimurium* infection induced extensive epithelial damage, submucosal edema, and an inflammatory cell infiltrate in GF mice harboring the microbiota from 4-day-old mice in the absence and presence of *Bacteroides* species (Fig. 3, B and C). *S. Typhimurium* infection did not induce any detectable epithelial damage or inflammatory pathology in GF mice colonized with the microbiota from 4-day-old

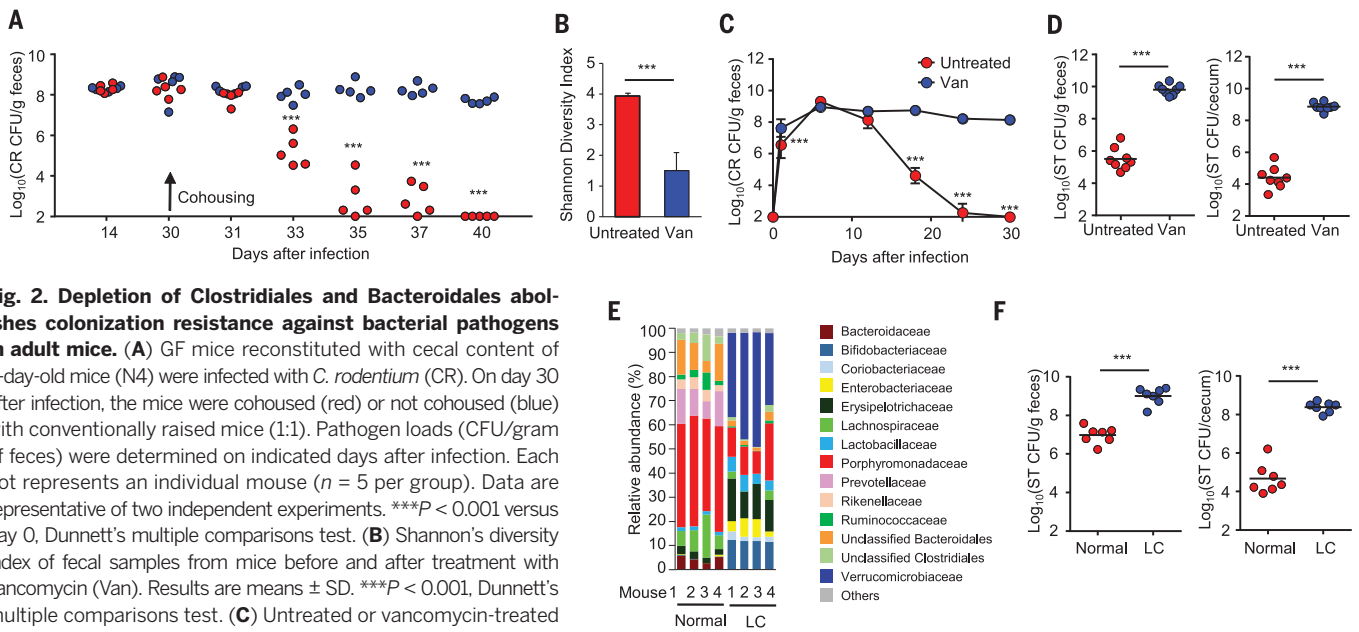
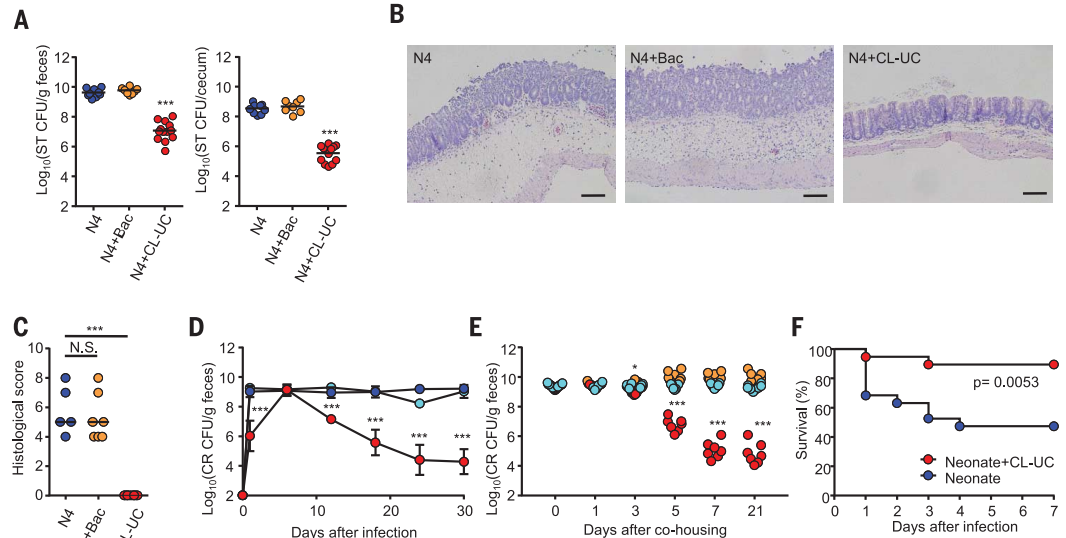


Fig. 2. Depletion of Clostridiales and Bacteroidales abolishes colonization resistance against bacterial pathogens in adult mice. (A) GF mice reconstituted with cecal content of 4-day-old mice (N4) were infected with *C. rodentium* (CR). On day 30 after infection, the mice were cohoused (red) or not cohoused (blue) with conventionally raised mice (1:1). Pathogen loads (CFU/gram of feces) were determined on indicated days after infection. Each dot represents an individual mouse ($n = 5$ per group). Data are representative of two independent experiments. $***P < 0.001$ versus day 0, Dunnett's multiple comparisons test. (B) Shannon's diversity index of fecal samples from mice before and after treatment with vancomycin (Van). Results are means \pm SD. $***P < 0.001$, Dunnett's multiple comparisons test. (C) Untreated or vancomycin-treated (Van) mice were infected with *C. rodentium* (CR). Pathogen loads (CFU/gram) in feces were determined on days 1, 6, 12, 18, 24, and 30 after infection. Results are means \pm SD and representative of two experiments ($n = 5$ per group). $***P < 0.001$ versus untreated, Sidak's multiple comparisons test. (D) Untreated or vancomycin-treated (Van) mice were infected with *S. Typhimurium* Δ spiA (ST). Pathogen loads in fecal (left) and cecal (right) contents were determined on day 1 after infection. Each dot represents an individual mouse ($n = 8$ per group). Data are pooled from two independent experiments. $***P < 0.001$, Mann-

Whitney U test. (E) Relative abundance of OTUs in fecal samples from adult mice fed a normal diet (Normal) or lactose-cellobiose-rich (LC) diet for 21 days. Colors correspond to families. (F) Adult mice were fed a normal or LC diet for 21 days and then were infected with *S. Typhimurium* Δ spiA (ST). Pathogen loads (CFU/gram) in fecal and cecal contents were determined on day 1 after infection. Each dot represents an individual mouse ($n = 7$ per group). Data are pooled from two independent experiments ($n = 3$ to 4 per group). $***P < 0.001$, Mann-Whitney U test.

Fig. 3. Administration of Clostridia, but not Bacteroides, restores colonization resistance to bacterial pathogens in the gut. (A to D) Adult GF mice reconstituted with microbiota of 4-day-old mice (N4, blue), N4 plus four *Bacteroides* species [Bac: *B. acidifaciens*, *B. thetaiotaomicron*, *B. vulgatus*, and *B. uniformis*] (N4+Bac, orange), or N4 plus *Clostridia* consortium (N4+CL-UC, red) were infected with *S. Typhimurium* Δ spiA (ST) and analyzed 1 day after infection. (A) Pathogen loads (CFU/gram) in fecal and cecal contents were determined by plating. Each dot represents an individual mouse (N4, $n = 9$; N4+Bac, $n = 8$; N4+CL-UC, $n = 13$). Data are pooled from three independent experiments. $***P < 0.001$ versus N4, Dunnett's multiple comparisons test. (B) Representative images of HE-stained cecal sections from N4, N4+Bac, and N4+CL-UC mice. Cecal tissue was processed 1 day after infection. Scale bars, 100 μ m. (C) Pathology scores of cecal tissue from GF reconstituted mice. Each dot represents an individual mouse (N4, $n = 5$; N4+Bac, $n = 7$; N4+CL-UC, $n = 10$). Data are pooled from three independent experiments. $***P < 0.001$; N.S., not significant; Dunnett's multiple comparisons test. (D) Adult GF mice (light blue) or GF mice reconstituted with 4-day-old microbiota (N4, dark blue) or N4 plus *Clostridia* consortium (N4+CL-UC, red) were infected with *C. rodentium* (CR). CFU/gram of feces were determined on days 1, 6, 12, 18, 24, and 30 after infection. Results are means \pm SD and representative of two experiments ($n = 4$ per group).



$***P < 0.001$ versus N4, Dunnett's multiple comparisons test. (E) GF mice reconstituted with N4 microbiota were infected with *C. rodentium* (CR). On day 30 after infection, the mice were cohoused with GF mice (GF, light blue) or GF mice reconstituted with four *Bacteroides* species (Bac, orange) or *Clostridia* consortium (CL-UC, red). CFU/gram of feces were determined in feces on days 0, 1, 3, 5, 7, and 21 after cohousing. Each dot represents an individual mouse (GF, light blue; $n = 7$), (Bac, dark blue; $n = 8$) and (CL-UC, red; $n = 13$). Data are pooled from three independent experiments. $*P < 0.05$; $***P < 0.001$ versus control, Dunnett's multiple comparisons test. (F) Ten-day-old littermate mice were left untreated (blue) or given *Clostridia* consortium (red) by gavage and then infected with *S. Typhimurium* Δ spiA (ST). Mouse survival was monitored over time after infection ($n = 19$ per treatment). $P = 0.0054$ by log-rank test.

$***P < 0.001$ versus GF mice, Dunnett's multiple comparisons test. (E) GF mice reconstituted with N4 microbiota were infected with *C. rodentium* (CR). On day 30 after infection, the mice were cohoused with GF mice (GF, light blue) or GF mice reconstituted with four *Bacteroides* species (Bac, orange) or *Clostridia* consortium (CL-UC, red). CFU/gram of feces were determined in feces on days 0, 1, 3, 5, 7, and 21 after cohousing. Each dot represents an individual mouse (GF, light blue; $n = 7$), (Bac, dark blue; $n = 8$) and (CL-UC, red; $n = 13$). Data are pooled from three independent experiments. $*P < 0.05$; $***P < 0.001$ versus control, Dunnett's multiple comparisons test. (F) Ten-day-old littermate mice were left untreated (blue) or given *Clostridia* consortium (red) by gavage and then infected with *S. Typhimurium* Δ spiA (ST). Mouse survival was monitored over time after infection ($n = 19$ per treatment). $P = 0.0054$ by log-rank test.

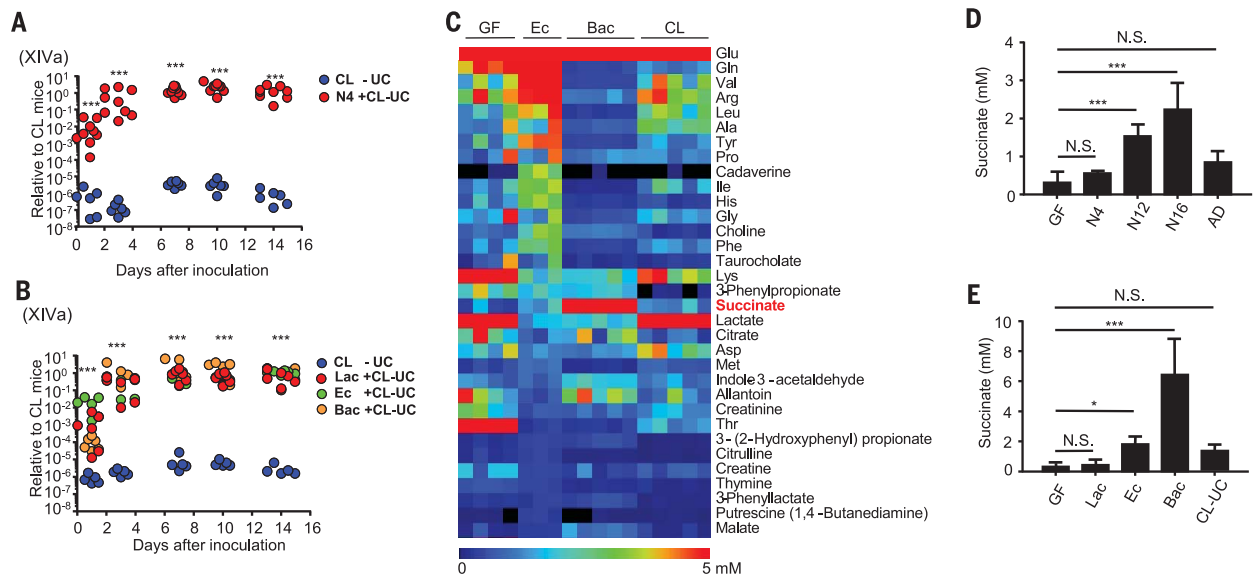


Fig. 4. The neonatal microbiota increases the abundance of protective *Clostridia* in the gut. (A) Analysis of the fecal microbiota from GF mice gavaged with *Clostridia* consortium (CL-UC) (blue, $n = 6$) or GF mice previously reconstituted with the cecal microbiota of 4-day-old mice and then gavaged with *Clostridia* consortium (N4+CL-UC; red, $n = 9$). The presence of *Clostridium* cluster XIVa in fecal DNA

was quantitated over time by qPCR. Results were normalized to the amounts of *Clostridium* cluster XIVa DNA in fecal samples from mice bearing the *Clostridia* consortium. Each dot represents an individual mouse. Data are pooled from three independent experiments. $***P < 0.001$ versus control, Sidak's multiple comparisons test. (B) Analysis of the fecal microbiota from GF mice gavaged with the *Clostridia* consortium (CL-UC, $n = 5$) or GF mice previously reconstituted with mouse *E. coli* (Ec+CL-UC, $n = 6$) or *B. acidifaciens* (Bac+CL-UC, $n = 6$). The presence of *Clostridium* cluster XIVa in fecal DNA was quantified by qPCR. Results were normalized to the amounts of *Clostridium* cluster XIVa DNA in fecal samples of *Clostridia* consortium. Each dot represents an individual mouse. Data are pooled from two independent experiments. $***P < 0.001$ versus Control, Sidak's multiple comparisons test. (C) Heat-map analysis of top 33 metabolites in fecal samples from GF or GF mice reconstituted with *E. coli* (Ec), *B. acidifaciens* (Bac), or *Clostridia* consortium (CL). (D) Succinate concentration in fecal samples from GF or GF mice reconstituted with cecal contents of 4-day-old (N4), 12-day-old (N12), 16-day-old (N16), or 7-week-old (AD) mice. Results are means \pm SD and representative of two experiments ($n = 4$ to 6 per group). $***P < 0.001$ versus GF mice, Dunnett's multiple comparisons test. (E) Succinate concentration in fecal samples from GF or GF mice reconstituted with *E. coli* (Ec), *L. murinus* (Lac), *B. acidifaciens* (Bac), or *Clostridia* consortium (CL-UC). Results are means \pm

mice and gavaged with *Clostridia* (Fig. 3, B and C). Similarly to observations on *S. Typhimurium*, the pathogen loads in the feces of GF mice, or of GF mice previously reconstituted with the microbiota of 4-day-old mice and then orally infected with *C. rodentium*, were reduced by 4 to 5 logs after administration of *Clostridia* (Fig. 3, D and E). No loss of *C. rodentium* was seen when a mixture of *Bacteroides* species was given to GF mice reconstituted with the microbiota of neonatal mice (Fig. 3, D and E).

We next asked whether host immunity plays a role in *Clostridia*-mediated colonization resistance against *S. Typhimurium* infection in the intestine. The microbiota from 4-day-old mice were transferred to wild-type, mutant GF mice deficient in Myd88/Trif, two essential adaptors for signaling via the Toll-like/interleukin-1 (IL-1)/IL-18 receptor family, or *Rag1*^{-/-} GF mice that are devoid of B and T cells. All these reconstituted GF mice exhibited unimpaired colonization resistance against *S. Typhimurium* infection

SD and representative of two experiments ($n = 4$ to 8 per group). $*P < 0.05$, $***P < 0.001$ versus GF mice, Dunnett's multiple comparisons test. (F) Fecal microbiota from GF mice reconstituted with *Clostridia* consortium was gavaged into untreated GF mice (Control) or mice treated with 100 mM succinate (Suc), 50 mM lactate (Lac), or 100 mM acetate (Ace) for 7 days. Gavage of *Clostridia* consortium was performed on day 7 after treatment, and metabolite administration was continued for another 14 days. The presence of *Clostridium* cluster XIVa in fecal DNA was monitored over time by qPCR. Each dot represents an individual mouse ($n = 6$ per group). Data pooled from two independent experiments. $***P < 0.001$ versus Control, Sidak's multiple comparisons test. (G) Untreated GF mice (Control) or GF mice treated with succinate (Suc) for 7 days were given *Clostridia* consortium by gavage. The mice were then infected with *S. Typhimurium* Δ spiA (ST), and pathogen loads in fecal and cecal contents were determined 1 day after infection by plating. Each dot represents an individual mouse (Untreated, $n = 9$; Suc, $n = 10$). Data are pooled from three independent experiments. $***P < 0.001$, Mann-Whitney *U* test. (H) GF mice were given regular drinking water (Mock) or treated with 100 mM succinate (Suc) in the drinking water for 7 days, and rectal oxygen concentration was determined. Each dot represents a mean oxygen concentration from two to four measurements for each mouse ($n = 4$). Data from three combined experiments. $*P < 0.05$, Mann-Whitney *U* test.

upon intragastric administration of *Clostridia* compared with GF mice that were not gavaged with *Clostridia* (fig. S5). Thus, colonization resistance against *S. Typhimurium* in the intestine does not require host stimulation via innate MyD88/Trif-regulated pathways or adaptive immunity. Certain antimicrobial proteins, including regenerating islet-derived 3 beta (Reg3 β) and IL-22-induced Reg3 γ , have been associated with colonization resistance to pathogens in some systems (13). Notably, the expression of Reg3 β , Reg3 γ ,

and *Il6*, but not *Muc2* or *Tnfa*, was higher in the cecum of GF mice colonized with the adult microbiota than in GF mice colonized with the microbiota of 4-day-old mice (fig. S6). However, the expression of *Reg3β* and *Reg3γ* was reduced in *Myd88^{-/-}Ticam^{-/-}* GF mice colonized with the adult microbiota (fig. S6). Likewise, the expression of *Il22*, a cytokine involved in the regulation of intestinal barrier function and *Reg3γ* (*14*), was reduced in the intestine of GF mice colonized with the microbiota of 4-day-old mice compared with that of adult mice (fig. S6). However, treatment with a neutralizing antibody to IL-22 to inhibit IL-22-mediated protection (*15*) neither affected *S. Typhimurium* loads in fecal and cecal contents nor influenced colon length in infected GF mice reconstituted with the microbiota of adult mice (fig. S7).

To determine whether *Clostridia* protected neonatal mice from pathogen challenge, 10-day-old mice were gavaged with the *Clostridia* consortium or left untreated and then intragastrically infected with the *S. Typhimurium* *ΔspiA* mutant. Notably, ~50% of the neonatal mice inoculated with *S. Typhimurium* succumbed to infection, whereas >90% of the neonatal mice previously gavaged with *Clostridia* survived (Fig. 3F). Collectively, these results indicate that *Clostridia* mediate colonization resistance against *S. Typhimurium* and *C. rodentium* via a mechanism that is independent of Myd88, Trif, B, and T cells. Furthermore, administration of *Clostridia* protects neonatal mice from mortality induced by pathogen challenge.

With the exception of a few Lachnospiraceae OTUs, which are present in the microbiota of 12-day-old mice, taxa in the order Clostridiales are absent from the microbiota of 4-day-old and 12-day-old mice but robustly colonize the intestine between days 12 and 16 of neonatal life, the time frame associated with the acquisition of colonization resistance against pathogens. To assess whether neonatal bacteria promote the colonization of *Clostridia* species, GF mice were first colonized with the microbiota from 4-day-old mice, and 7 days later they were gavaged with the *Clostridia* consortium. The abundance of *Clostridium* IV and XIVa clusters, which constitute the predominant *Clostridia* in the consortium assessed by quantitative polymerase chain reaction (qPCR), was low after intragastric gavage to GF mice (Fig. 4A and fig. S8A). In the presence of the 4-day-old neonatal microbiota, the intestinal colonization of *Clostridia* increased by ~6 logs (Fig. 4A and fig. S8A). Thus, colonization of *Clostridia* is reduced in the absence of neonatal bacteria. However, if GF mice were reconstituted with the microbiota of 4-day-old

mice, then subsequent intragastric administration of *Clostridia* induced robust colonization resistance against *S. Typhimurium* (fig. S9). Likewise, preinoculation of GF mice with *Lactobacillus murinus* or *E. coli*, species that are present in 4- and 12-day-old neonatal microbiota, respectively, or with *Bacteroides acidifaciens* whose colonization coincides with robust acquisition of Clostridiales in the microbiota of 16-day-old mice, enhanced the colonization of *Clostridia* by 5 to 6 logs (Fig. 4B and fig. S8B).

To assess whether bacteria-derived metabolites regulate intestinal colonization by *Clostridia*, we performed unbiased capillary electrophoresis-time-of-flight mass spectrometry (CE-TOFMS)-based metabolome analysis of the cecal contents of GF mice and GF mice colonized with dominant bacterial species present in the ceca of neonatal and adult mice. The metabolome analysis revealed that amounts of succinate were very low in the cecal contents of GF mice. Succinate levels were also low in GF mice reconstituted with *Clostridia*, slightly higher in GF mice colonized with *E. coli*, and significantly elevated in GF colonized with *Bacteroides* when compared with GF mice (Fig. 4, C and D). Succinate levels were increased in GF mice reconstituted with the microbiota of 12- and 16-day-old mice, but not in those given microbiota of 4-day-old mice or given lactobacilli (Fig. 4, D and E), indicating that an increase in succinate levels is not required for *Clostridia* colonization. Administration of succinate, but not acetate or lactate, in drinking water did, however, enhance colonization of *Clostridia* belonging to the dominant IV and XIVa clusters by 4 to 5 logs (Fig. 4F and fig. S8C). Consistent with these results, succinate in the drinking water reduced the intestinal loads of *S. Typhimurium* *ΔspiA* by ~100-fold in GF mice given the *Clostridia* consortium by gavage (Fig. 4G). Aerobic and facultative anaerobic bacteria have been suggested to consume oxygen in the distal intestine, which then promotes the colonization of strict anaerobes (*16*). We found that succinate administration did reduce the concentration of oxygen in the intestine of GF mice (Fig. 4H). Together, these results indicate that the neonatal microbiota contribute to the acquisition of protective *Clostridia* before weaning and is a critical event that prevents the growth of enteric pathogens in the gut early in life.

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SUPPLEMENTARY MATERIALS

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Material and Methods
Figs. S1 to S9
References (17–19)

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Neonatal acquisition of *Clostridia* species protects against colonization by bacterial pathogens

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Gut anaerobes protect against pathogen invasion

Intestinal infections are a common problem for young animals. One explanation is that the protective gut microbiota is not fully established in infants. How the microbiota might protect against pathogens is unclear. Kim *et al.* found that members of the group of strictly anaerobic, spore-forming bacteria known as clostridia protect neonatal mice against diarrhea-causing pathogens. The protective effect is enhanced by giving mice the metabolite succinate in drinking water. Succinate favors colonization of the neonatal gut by cluster IV and XIVa clostridia and concomitantly excludes *Salmonella typhimurium*.

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