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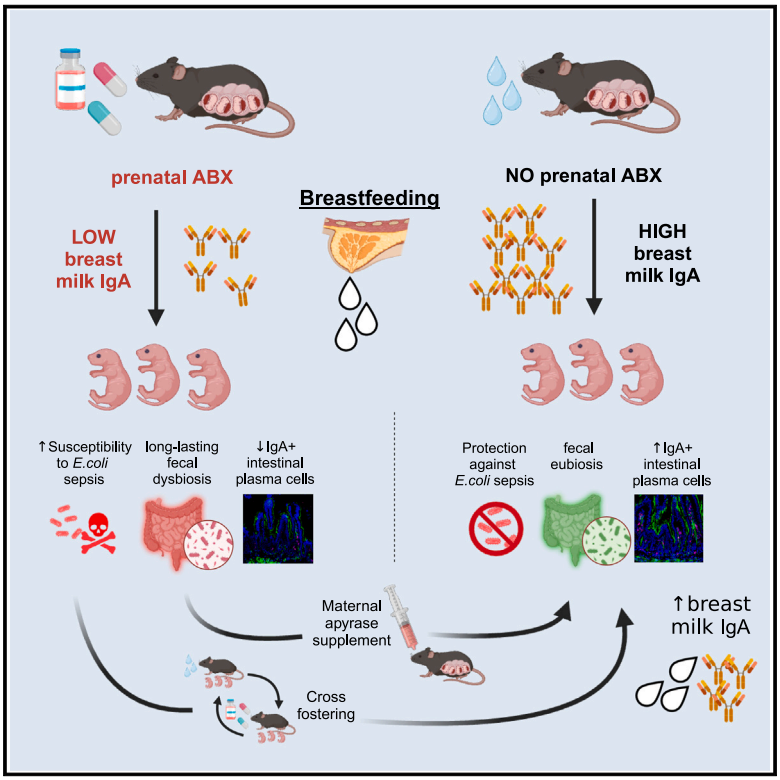
Prenatal antibiotics reduce breast milk IgA and induce dysbiosis in mouse offspring, increasing neonatal susceptibility to bacterial sepsis

Graphical abstract



Highlights

- Prenatal antibiotic treatment renders pups more susceptible to late-onset *E. coli* sepsis
- Pups born to antibiotic-treated mothers have reduced fecal SIgA and IgA-coated bacteria
- SIgA from breast milk and the absence of dysbiosis protect mice from late-onset sepsis
- Prenatal antibiotic treatment has long-term effects on the neonatal gut immune system

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In brief

Up to one-third of women receive antibiotics during pregnancy. Pietrasanta et al. show that antibiotic treatment of pregnant mice reduces the amount of breast milk antibodies (SIgA) and alters the neonatal fecal microbiota, making neonatal mice more susceptible to sepsis caused by harmful bacteria that can translocate from the gut.

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Prenatal antibiotics reduce breast milk IgA and induce dysbiosis in mouse offspring, increasing neonatal susceptibility to bacterial sepsis

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SUMMARY

Antibiotics (Abx) are administered to 20%–30% of pregnant women, but their effects on neonatal immune development are poorly understood. We show that newborn mice born to Abx-treated dams are more susceptible to late-onset sepsis. This susceptibility is linked to lower maternal breast milk immunoglobulin A (IgA), neonatal fecal IgA, and IgA coating of intestinal bacteria, thus causing the translocation of intestinal pathobionts. Weaned young adults born to Abx-treated mothers had reduced IgA+ plasma cells in the ileum and colon, fecal secretory IgA (SIgA), colonic $CD4^+$ T regulatory lymphocytes and T helper 17-like lymphocytes, and a less diverse fecal microbiome. However, treatment with apyrase, which restores SIgA secretion, prompted IgA production in breast milk and protected pups from sepsis. Additionally, breast milk from untreated mothers rescued the phenotypes of pups born to Abx-treated mothers. Our data highlight the impact of prenatal Abx on breast milk IgA and their long-term influence on intestinal mucosal immune function mediated by breastfeeding.

INTRODUCTION

Early life is a period of rapid and massive transformations as no other in the life of mammalians. Both before and soon after birth, the systemic and mucosal immune system undergo profound anatomic and functional modifications that are significantly influenced by the assembling neonatal microbiome. The relationship between the neonatal immune system and the microbiome is reciprocal, and any perturbation to each of these elements affects the development of the other.[1,2] In the gut, this connection is the tightest. It has been shown that the different species and abundance of intestinal microbiome can shape the development of key components of the intestinal mucosal immune system, such as antimicrobial peptides (AMPs),[3] dendritic cells (DCs),[4–6] innate lymphoid cells (ILCs),[7] and antigen-specific B and T lymphocytes residing in the lamina propria (LP) of the small intestine (SI). An immune feature greatly conditioned by the presence of a physiological microbiome is the production of secretory immunoglobulin A (SIgA) by the Peyer's patches (PPs) and by LP plasma cells,[8] which can in turn favor the selection of commensal species, protect against translocation of pathogenic bacteria into the bloodstream, and neutralize bacterial toxins.[9] In early life, SIgA abundance in the gastrointestinal tract is mostly determined by passive acquisition from maternal breast milk, whereas the functionality of SI IgA+ plasma cells becomes significant after the transition to solid food diet.[10] Breast milk SIgA are critical for the protection against late-onset neonatal sepsis (LOS) and, as recently demonstrated, necrotizing enterocolitis (NEC).[11] Therefore, especially in neonates, a tight, triple, mutual connection exists between nutrition, the assembling

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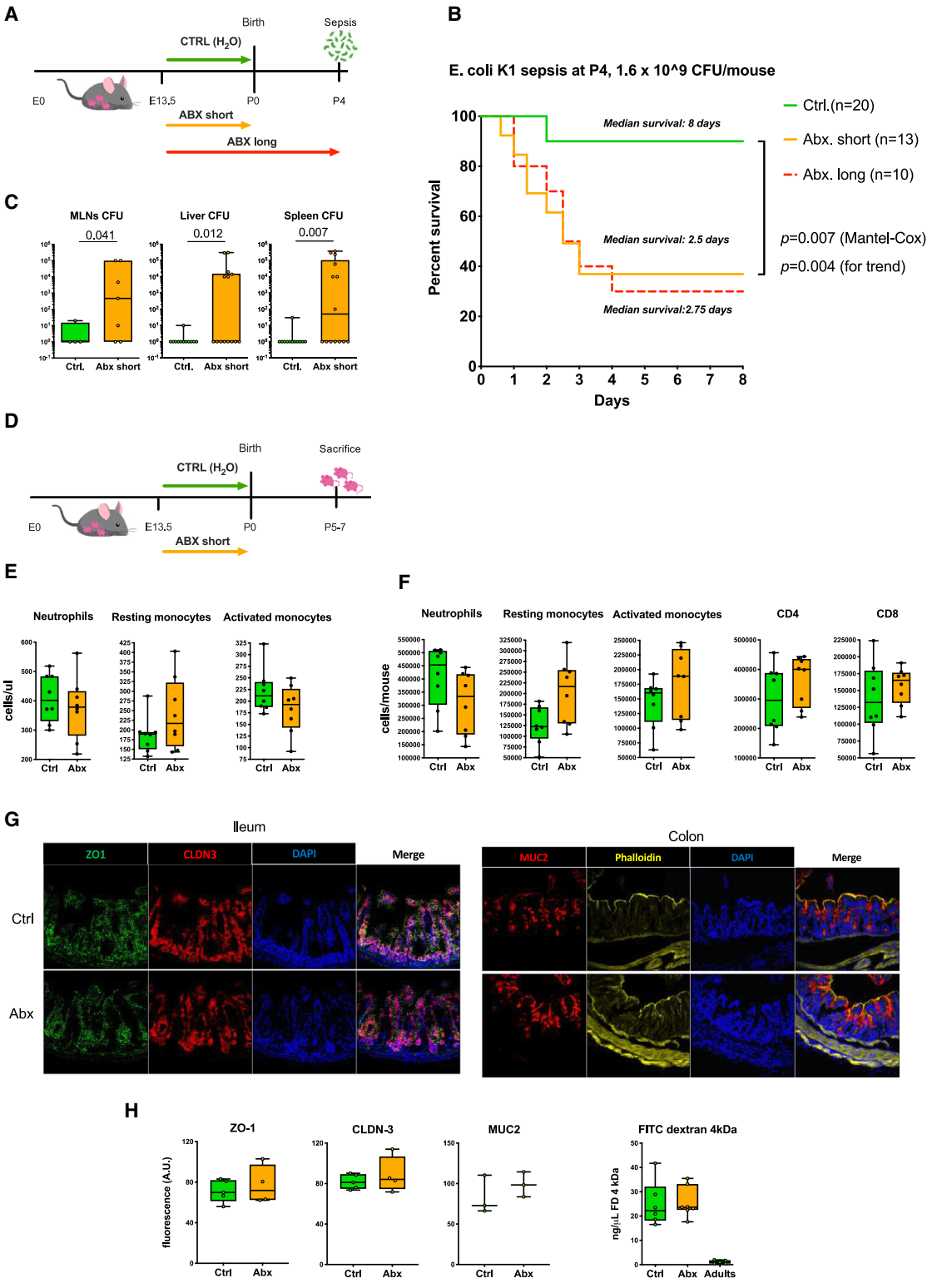

Figure 1. Prenatal antibiotics increase neonatal susceptibility to *E. coli* sepsis without altering systemic immunity or intestinal epithelial structure

(A) Experimental layout for *E. coli O18:K1* late-onset neonatal sepsis challenge.

(B) Survival curves of mice after LOS induced by *E. coli K1*. Pooled results from 3 independent experiments.

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microbiome, and gut immune system. Antibiotics (Abx) are prescribed to 20%–30% of pregnant women, representing the drugs most frequently administered during pregnancy.[12,13] The main reasons for prescription are the treatment of prelabor (especially preterm) rupture of membranes (PROMs), suspected intrauterine infection (IUI), prophylaxis for group B Streptococcus colonization of the birth canal, or urinary tract infections. Thus, for a large part of neonates, especially those born prematurely, mothers have been treated with Abx during pregnancy. In animal models, it has been shown that pups exposed to Abx in early life might be more susceptible to LOS due to granulocytopenia, caused by intestinal dysbiosis that hampers interleukin (IL)-17 production by intestinal group 3 ILCs.[14] Alternatively, an unbalanced intestinal microbiome in early life can favor neonatal LOS per se, depending on the expansion or reduction of specific bacterial species.[15] However, these evidence on animal models are not mirrored by robust evidence in humans,[16] possibly because of constitutional differences between human and murine microbiota-immune system interactions,[17,18] but also because animal models rarely distinguish between Abx administered exclusively during pregnancy and those administered during lactation, grouping them together under the label of "early life" exposure. Here, we evaluated the effects of maternal Abx administered exclusively before delivery on neonatal systemic and gastrointestinal immune function, focusing on the susceptibility to LOS caused by translocation of gram-negative bacteria into the bloodstream. We reveal that Abx greatly reduced the production of IgA by the maternal mammary gland after birth and that the combination of low breast milk IgA and altered neonatal intestinal microbiome can negatively imprint the development of IgA-producing plasma cells in the neonatal intestine for long, even after the interruption of breastfeeding.

RESULTS

Effects of prenatal Abx on neonatal systemic immunity, intestinal structure, and susceptibility to *E. coli* sepsis

To mimic the exposure of pregnant women to antimicrobials during the last trimester of pregnancy, we administered to pregnant C57BL/6 dams a mixture of three Abx commonly used during human pregnancy ("Abx": ampicillin, a broad-spectrum penicillin; neomycin, an aminoglycoside; and erythromycin, a macrolide) in drinking water between embryo day 13.5 (E13.5) and E18.5. The general experimental model is represented in Figure 1D. Prenatal Abx rapidly caused a significant reduction in the bacterial load of maternal fecal pellets, affecting both aerobe and anaerobe species (Figure S1A), a visible enlargement of the cecum, filled with intestinal mucus, and the elongation of SI (Figures S1B and S1C). Abx-induced dysbiosis was also matched by a reduction in the transcription of several antimicrobial proteins and peptides by maternal SI wall cells, such as regenerating islet-derived protein 3 gamma (Reg3γ) and defensin alpha-5 (Defa-5), and by an almost significant increase in the transcription of the mucin gene mucin-2 (MUC2) (Figure S1D). Despite these clear functional modifications, the epithelial and endothelial structures of maternal SI did not seem altered by Abx treatment. Indeed, confocal microscopy imaging (Figures S1E and S1F) did not demonstrate significant alterations in the localization or quantitative expression of neither epithelial (zonula occludens-1 [ZO-1]) nor endothelial (plasmalemma-vesicle-associated protein [PLVAP])[19] barrier proteins, and the quantification of mRNA levels (Figure S1H) of epithelial tight junctions (TJs)—associated proteins (ZO-1 and occludin [OCLN]) and of the endothelial gut-vascular barrier (GVB) was also not affected. Accordingly, a fluorescein isothiocyanate (FITC)-dextran (4 kDa) functional assay to evaluate the intestinal permeability to small molecules did not demonstrate a clear alteration in plasma FITC-dextran concentration 4 h after administration by oral gavage (Figure S1G). Thus, Abx treatment during the last 5 days of pregnancy in C57BL/6 mice profoundly altered the microbial content of maternal intestine and the excretion of antimicrobial proteins and peptides in the lumen, without major perturbation of the permeability of SI wall, the structure of intestinal epithelium or of the GVB.

To gain a more comprehensive and functional insight into the consequences of prenatal Abx therapy on neonatal immune response to infection, we investigated the susceptibility of pups either exposed or not to prenatal Abx to an experimental model of LOS, challenging 4- to 5-day-old pups (P4–P5) with a high dose of live *Escherichia coli* (*E. coli*) serotype O18:K1 administered by gastric gavage (Figure 1A). Pups born to mothers not exposed to Abx during pregnancy (Figure 1B, green line) were relatively resistant to *E. coli* challenge, with a mortality rate of 10% and a median survival exceeding the pre-established length of the experiment (8 days). Conversely, the mortality rate of pups exposed to Abx only during the fetal life (Figure 1B, yellow line) was 63%, with a median survival of 2.5 days. Considered previously published literature,[14,15] we also added an experimental control group of pups challenged with *E. coli* O18:K1 after exposure to Abx both pre- and post-natally (Figure 1B, red dotted line): these pups showed a median survival of 2.75 days, and mortality rates closely resembling that of pups with prenatal-only exposure. Therefore, we focused further experiments on pups exposed to Abx exclusively before

(C) Quantification of *E. coli* colonies (colony-forming units [CFUs]) 48 h after gavage in peripheral organs. MLNs, mesenteric lymph nodes. n = 5–8 mice per group, representative of at least 2 independent experiments. p values calculated by t test.
(D) General experimental layout.
(E) Absolute amount of circulating myeloid cells at P5 in pups born to Abx-treated or untreated mothers. n = 5–8 mice per group, representative of 2 independent experiments. p values calculated by t test.
(F) Absolute amount of spleen myeloid and lymphoid cells at P5 in pups born to Abx-treated or untreated mothers. n = 5–8 mice per group, representative of 2 independent experiments. p values calculated by t test.
(G) Confocal microscopy images of neonatal ileum and colon at P7 without or with prenatal Abx treatment. ZO-1, zonula occludens-1; CLDN-3, claudin 3; MUC2, mucin-2.
(H) Quantification of fluorescence in ileum (ZO-1, CLDN-3) and colon (MUC2), and serum concentration of 4 kDa FITC-dextran 4 h after gavage in pups at P7. n = 5–8 mice per group, representative of 2 independent experiments. p values calculated by t test. For (C), (E), (F), and (H), whiskers indicate minimum and maximum values.

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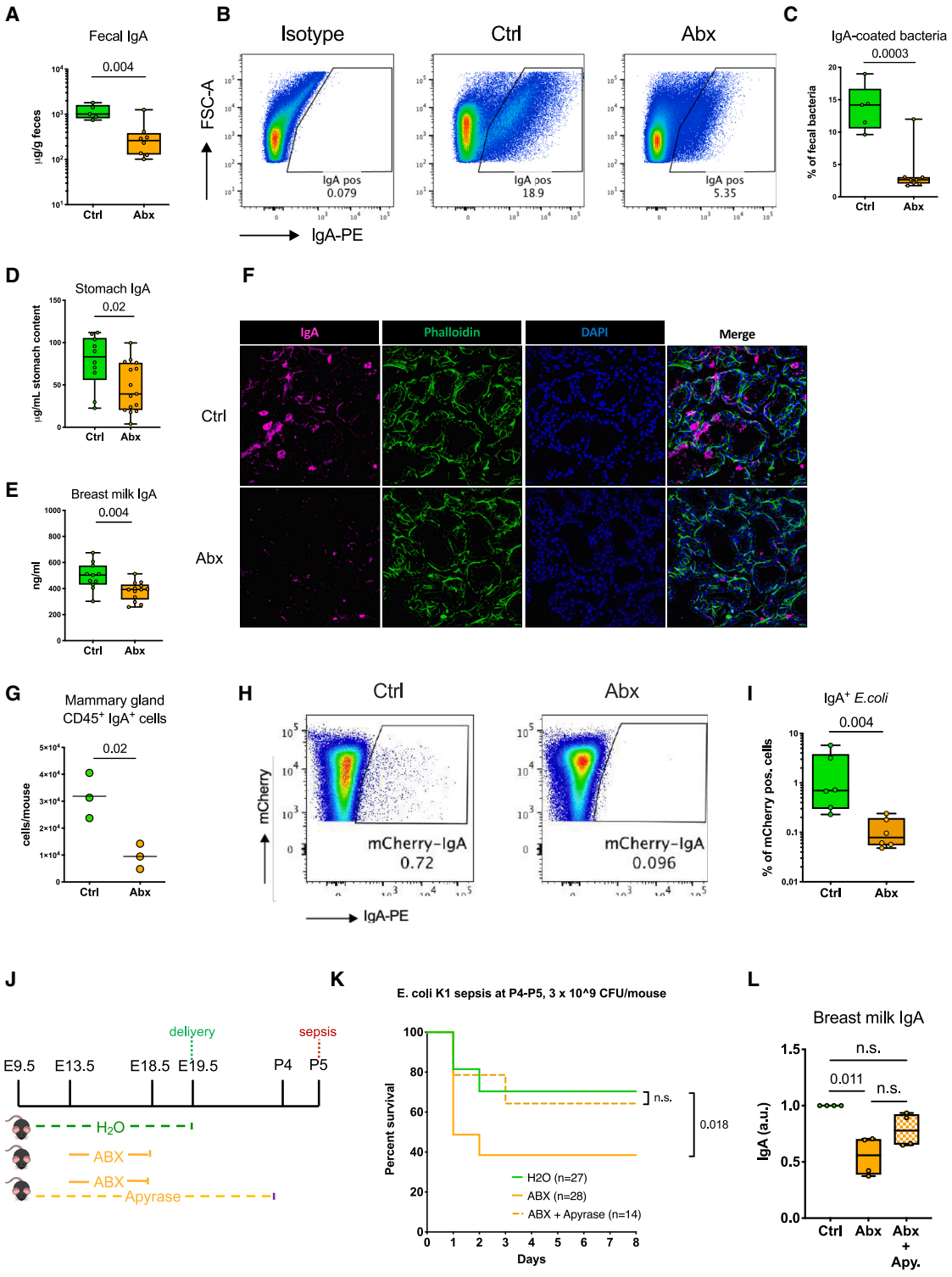

Figure 2. Prenatal antibiotics reduce neonatal fecal IgA, breast milk IgA, and IgA production by the maternal mammary gland

(A) Quantification of fecal IgA at P7 in pups born to untreated or Abx-treated mothers. *n* = 5–8 mice per group, representative of at least 2 independent experiments. *p* values calculated by t test.

(B) Representative dot plots of FACS analysis applied to identify IgA-coated bacteria in fecal pellets.

(C) Percentage of fecal bacteria coated by IgA in the two experimental groups. *n* = 5–8 mice per group, representative of at least 2 independent experiments. *p* values calculated by t test.

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delivery. Their significantly increased susceptibility to LOS correlated with a higher translocation of *E. coli* to distant organs such as mesenteric lymph nodes (MLNs), liver, and spleen (Figure 1C).

Available experimental evidence suggests that the exposure of neonatal mice to antimicrobials in early life can alter several aspects of immune function. Nonetheless, most experimental models apply prolonged Abx exposure both before and after birth, or non-clinically relevant Abx cocktails, including the oral administration of vancomycin, which is rarely used during human pregnancy.[14,15] With our experimental model we detected no significant alteration at 5 days after birth (P5) in blood (CD45+/CD11b+/Ly6Cint/Ly6G+ neutrophils, CD45+/CD11b+/Ly6Clow/Ly6G− resting monocytes and CD45+/CD11b+/Ly6-Chi/Ly6G− activated monocytes, Figure 1E) or spleen (neutrophils, resting and activated monocytes, CD45+/CD11b−/CD3+/CD4+ and CD8+ lymphocytes, Figure 1F) immune populations. Thus, differently from Abx treatment before and after birth,[14] prenatal, short-term Abx treatment of pregnant mothers is not sufficient to induce quantitative modifications of the systemic immune compartment in the P5 offspring. We then investigated whether the exposure to prenatal Abx could alter the intestinal structure and permeability of the offspring, justifying an increased bacterial translocation into the bloodstream. By confocal fluorescence microscopy and mRNA quantification at P5, we detected no significant difference between pups exposed or not to prenatal-only Abx in the expression of epithelial TJ proteins (ZO-1 and claudin 3), in the production of mucus protein (MUC2) and in the amount of fluorescent dextran (4 kDa FITC-dextran) retrievable from serum 4 h after gavage (Figures 1G and 1H). These results underlined how immune alterations induced by Abx-dependent dysbiosis are strictly dependent on the experimental conditions.

Prenatal Abx exposure reduces breast milk and neonatal gastrointestinal IgA levels

The increased susceptibility to bacterial translocation and LOS of pups exposed to Abx *in utero*, despite the apparent lack of significant alterations in the systemic immune compartment or in the structure of intestinal epithelium, led us to focus on other aspects of the neonatal intestinal immune system. Maternal breast milk is a key contributor to intestinal immune function during the neonatal period.[20] Moreover, it is known that SIgA coat and contain the commensal and pathogenic microbiota and that, in turn, the latter can stimulate and shape the production of SIgA.[8,21,22] Therefore, we reasoned that prenatal Abx treatment might affect the amount of SIgA in the neonatal gastrointestinal tract. Indeed, total fecal IgA at 1 week of life were significantly reduced in pups exposed to Abx *in utero* (Figure 2A). This corresponded to a drastically lower proportion of fecal bacteria coated by the same IgA, as assessed by flow cytometry, in pups born to dams exposed to Abx (Figures 2B and 2C). We next evaluated the amount of total IgA in the stomach content of sacrificed pups, as a reliable proxy for maternal breast milk. Pups exposed to Abx had significantly lower amounts of IgA in their stomach (Figure 2D). We also directly quantified IgA in breast milk of lactating dams and analyzed the mammary glands of mothers exposed or not to prenatal Abx by flow cytometry and confocal fluorescent microscopy. As highlighted in Figures 2E–2G, dams exposed to prenatal Abx excreted significantly lower amounts of IgA in the breast milk and had significantly reduced CD45+IgA+ mammary plasma cells 1 week after the interruption of Abx, supporting the evidence that maternal intestinal dysbiosis caused by Abx correlates with a long-lasting reduction of breast milk SIgA. To address whether there were polyreactive SIgA, we generated a fluorescently labeled *E. coli* K1 by electroporation using pONmCherry plasmid and orally gavaged neonatal mice at P5 with it. We confirmed the presence of polyspecific SIgA capable to bind to *E. coli* K1 in the neonatal fecal pool and a significantly lower proportion in pups born to dams exposed to prenatal Abx (Figures 2H and 2I).

To evaluate the contribution of SIgA in the protection against *E. coli* K1, we artificially increased the level of SIgA in pregnant dams subjected to Abx. Extracellular ATP in the SI lumen limits the generation of SIgA by inhibiting T follicular helper (Tfh) cells activity and development of IgA-secreting cells in PPs. Treatment of mice with apyrase, an ATP-hydrolyzing enzyme, is sufficient to enhance SIgA excretion in the SI.[23,24] We therefore treated pregnant mothers receiving prenatal Abx with recombinant apyrase,[23] from E9.5 to P4 after delivery (Figure 2J). Pups born to mothers treated with prenatal Abx and supplemented with apyrase were rescued from the consequences of *E. coli* K1 sepsis (Figure 2K), and breast milk of their mothers trended toward increased IgA as compared with mothers treated with prenatal Abx only (Figure 2L).

Thus, coating by passively transferred IgA in the gastrointestinal lumen is critical for protection against pathogen translocation in the context of neonatal LOS and is reduced after maternal exposure to Abx during pregnancy. Protection is restored upon apyrase in pregnant dams.

(D) Quantification of stomach IgA at P7 in the two experimental groups. n = 5–8 mice per group, representative of at least 2 independent experiments. p values calculated by t test.
(E) Quantification of breast milk IgA at P7 in the mothers of the two experimental groups. n = 5–8 mice per group, pooled from at least 2 independent experiments. p values calculated by t test.
(F) Confocal microscopy images of the mammary gland of Abx-untreated or -treated dams at P7. n = 3 mice per group, representative of 2 independent experiments.
(G) Absolute number of CD45+ IgA+ live cells in the mammary gland of Abx-untreated or -treated dams at P7. n = 5–8 mice per group, representative of at least 2 independent experiments. p values calculated by t test.
(H) Gating strategy used to identify IgA-coated fluorescent *E. coli*.
(I) Quantification of the IgA coating of fluorescent *E. coli K1* in fecal pellets 24 h after gavage. n = 6 mice per group, representative of 2 independent experiments. p *values* calculated by t test.
(J) Experimental layout for maternal supplementation with purified apyrase (APY) and subsequent *E. coli K1* late-onset neonatal sepsis challenge.
(K) Survival curves of mice after LOS induced by *E. coli* K1. Pooled results from 2 independent experiments.
(L) Normalized concentration of breast milk IgA at P5 in the mothers of the three experimental groups. n = 4 dams per group, pooled from 2 independent experiments. p values calculated by Kruskal-Wallis test with Dunn's correction for multiple comparisons. For (A), (C), (D), (E), (I), and (L), whiskers indicate minimum and maximum values.

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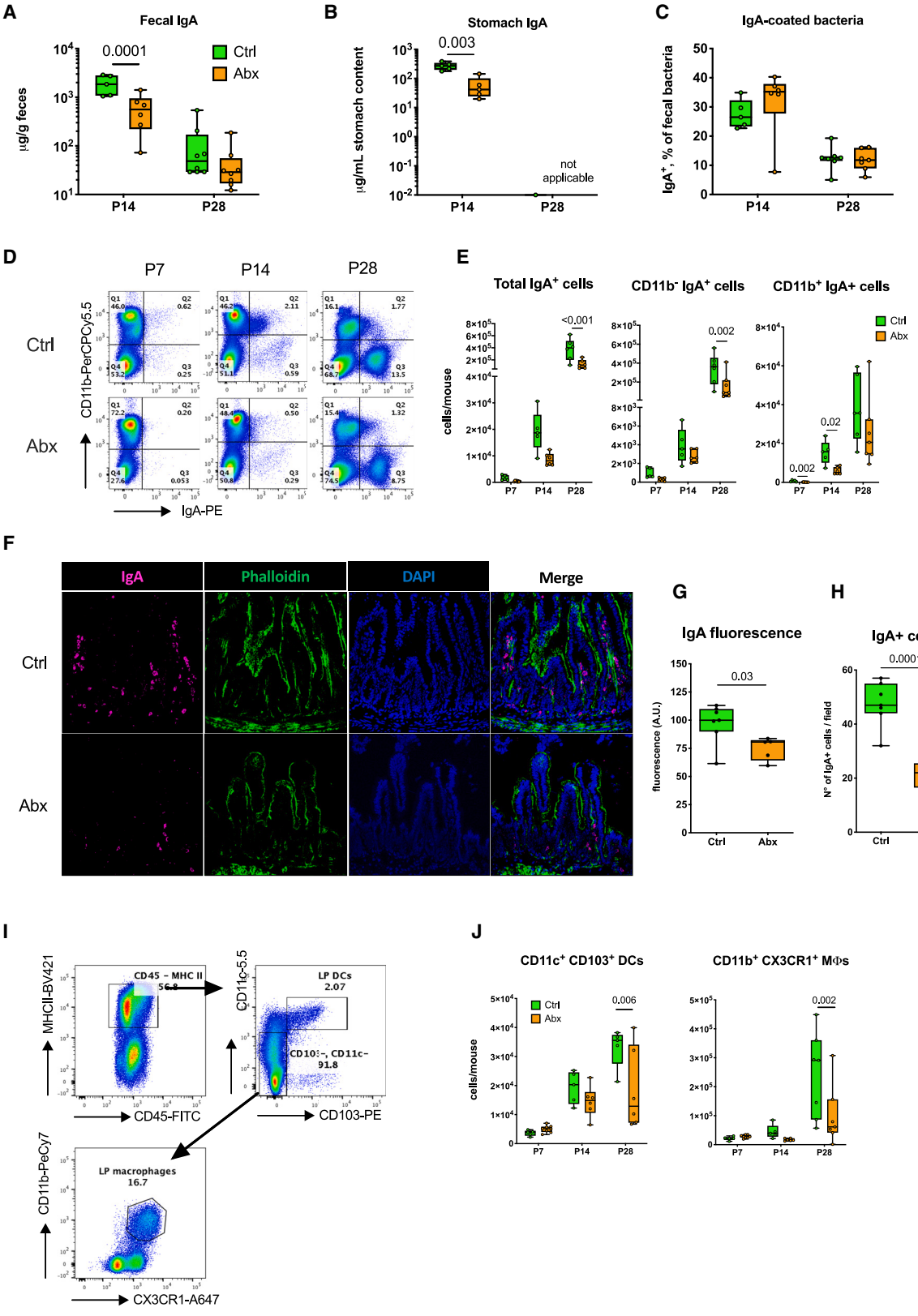

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Long-term effect of prenatal Abx on IgA production by plasma cells of the SI LP

Any perturbation to the physiological establishment of immune functions during early life might have an impact extending beyond the neonatal period.[25,26] Furthermore, the maturation of the gastrointestinal immune system in mammals is supported and stimulated by the encounter with food antigens,[27] which in mice occurs between the second and the third post-natal week. We thus analyzed longitudinally the effect of prenatal Abx treatment on pups, either before (P7–P14) or after (P28) the introduction of solid food. The absolute amount of fecal IgA in mice exposed to prenatal Abx was significantly lower, as compared with unexposed pups, at 14 days of life (P14), and remained, but not significantly, lower even at 28 days of life (Figure 3A). Similarly, the amount of stomach IgA was also reduced at P14 (Figure 3B), indicating that breastmilk was still representing a significant source of luminal IgA for the pups. Conversely, the proportion of fecal bacteria coated by these IgA did not mirror the kinetic of IgA amounts, being almost overlapping at both P14 and P28 between pups exposed or not to prenatal Abx (Figure 3C). Most luminal SIgA within the gastrointestinal tract of adult mice are produced in the ileum by CD45+ CD19− CD138+ plasma cells residing in the LP and in PP.[28] Thus, we next quantified the IgA-producing plasma cells in the ileum LP of neonatal mice at different post-natal ages with or without exposure to prenatal Abx, by multiparameter flow cytometry and confocal microscopy, to evaluate their contribution to the total pool of luminal SIgA. Among CD45+ IgA+ plasma cells, we separately evaluated the two subsets of CD11b+ and CD11b− plasma cells, as they can have different functional correlation with the intestinal microbiome.[29] In control pups, IgA+ plasma cells in the ileum LP at P7 were very limited, composing less than 1% of CD45+ cells. At P14, with the exposure to solid food antigens, the proportion of IgA+ cells increased significantly compared with P7, with a temporary wave of CD45+ IgA+ CD11b+ cells. Then, at the age of P28, the proportion of IgA+ LP plasma cells was significantly higher, with a clear prevalence of CD11b− cells (Figure 3D). When compared with control mice, pups exposed to prenatal Abx presented lower amounts of total CD45+ IgA+ plasma cells at both P14 and P28, with a statistically significant reduction in CD11b− cells at P28, and of CD11b+ cells at P7 and P14 (Figure 3E). This quantitative reduction was confirmed by confocal microscopy at P28 (Figures 3F–3H). Similar trends were observed in colonic LP IgA-secreting plasma cells, which were reduced both in absolute number and proportion of CD45+ cells in pups born to Abx-treated dams (Figures S2A and S2B), but their contribution to the total pool of fecal IgA was estimated as modest, given their extremely lower absolute number as compared with that of the SI. Confocal microscopy imaging corroborated the fluorescence-activated cell sorting (FACS) data, showing reduced amount of colonic IgA+ LP cells in pups exposed to prenatal Abx (Figures S2C and S2D). We also quantified the amount of CD11b+ CD11c+ CD103+ DCs and of CD11b+ CX3CR1+ CD11c− mucosal macrophages (MΦs) at different post-natal ages in pups exposed or not to prenatal Abx (Figures 3I–3L). Both at P14 and, statistically significant, at P28, pups born to Abx-treated dams had lower amounts of intestinal DCs, as well as lower amounts of intestinal MΦs at P28 (Figure 3L). These findings collectively suggested that prenatal Abx can have long-lasting effects on the pool of neonatal intestinal SIgA and on several immune populations, including DCs, MΦs, and plasma cells in the neonatal ileal LP.

Prenatal Abx reduce neonatal intestinal Th17 cells

It has been shown that maternal colonization/infection with gram-negative intestinal bacteria increases long-term the number of T helper-17 lymphocytes[30] and of ILC3.[31] Both of these cell types support the maturation of mucosal lymphoid organs and participate in antimicrobial defense through the production of IL-17 and IL-22.[32] Therefore, we hypothesized that maternal Abx-induced dysbiosis during pregnancy could also modify long-term these cell populations in weaned offspring (4 weeks old). As shown in Figures 4A–4C, prenatal Abx treatment affected the absolute number of RAR-related orphan receptor-γ (RORγt)+ ILC3 and the frequency (among CD4+ T cells) of Treg cells (Tregs) in the colon and significantly reduced the absolute number and the frequency (among CD4+ T cells) of T helper 17 lymphocyte (Th17) cells, in both ileal and colonic LP. Thus, prenatal Abx treatment of pregnant mothers can also negatively affect both the innate and adaptive lymphoid compartment of gastrointestinal mucosal immune system.

Effect of prenatal Abx on neonates is dependent on breastfeeding

To gain insight into how prenatal and post-natal events could affect the offspring in the context of prenatal Abx therapy, we

Figure 3. Maturation of intestinal immunity is altered in neonates exposed to prenatal antibiotics

(A) Fecal IgA at different post-natal ages in pups born to untreated (Ctrl) or Abx-treated (Abx) dams. n = 6–15 mice per group, representative of at least 2 independent experiments. p values calculated by two-way ANOVA with Sidak correction for multiple comparisons.

(B) Stomach IgA in the same experimental conditions of (A).

(C) IgA coating of fecal bacteria in the same experimental conditions of (A).

(D) Representative dot plots of CD11b-positive or -negative plasma cells in the lamina propria (LP) of neonatal ileum, at different post-natal ages, in the two experimental groups.

(E) Quantification of total IgA+ plasma cells and of CD11b+/− subpopulations of plasma cells at different post-natal ages in the two experimental groups. n = 5–8 mice per group, representative of at least 3 independent experiments. p values calculated by two-way ANOVA with Sidak correction for multiple comparisons.

(F) Confocal microscopy images of neonatal ileum at P28, in the two experimental groups. Representative of 7–8 mice/group.

(G) Quantification of IgA fluorescence in (F). p value calculated by t test.

(H) Number of IgA-fluorescent cells per field of view in (F). p value calculated by t test.

(I) Gating strategy used to identify dendritic cells (DCs) and macrophages (MΦs) in the ileum LP of pups born to Abx-untreated or -treated mothers.

(J) Total DCs and MΦs in the LP at different post-natal ages in the two experimental groups. n = 5–8 mice per group, representative of at least 3 independent experiments. p values calculated by two-way ANOVA with Sidak correction for multiple comparisons. For (A), (B), (C), (E), (G), (H), and (L), whiskers indicate minimum and maximum values.

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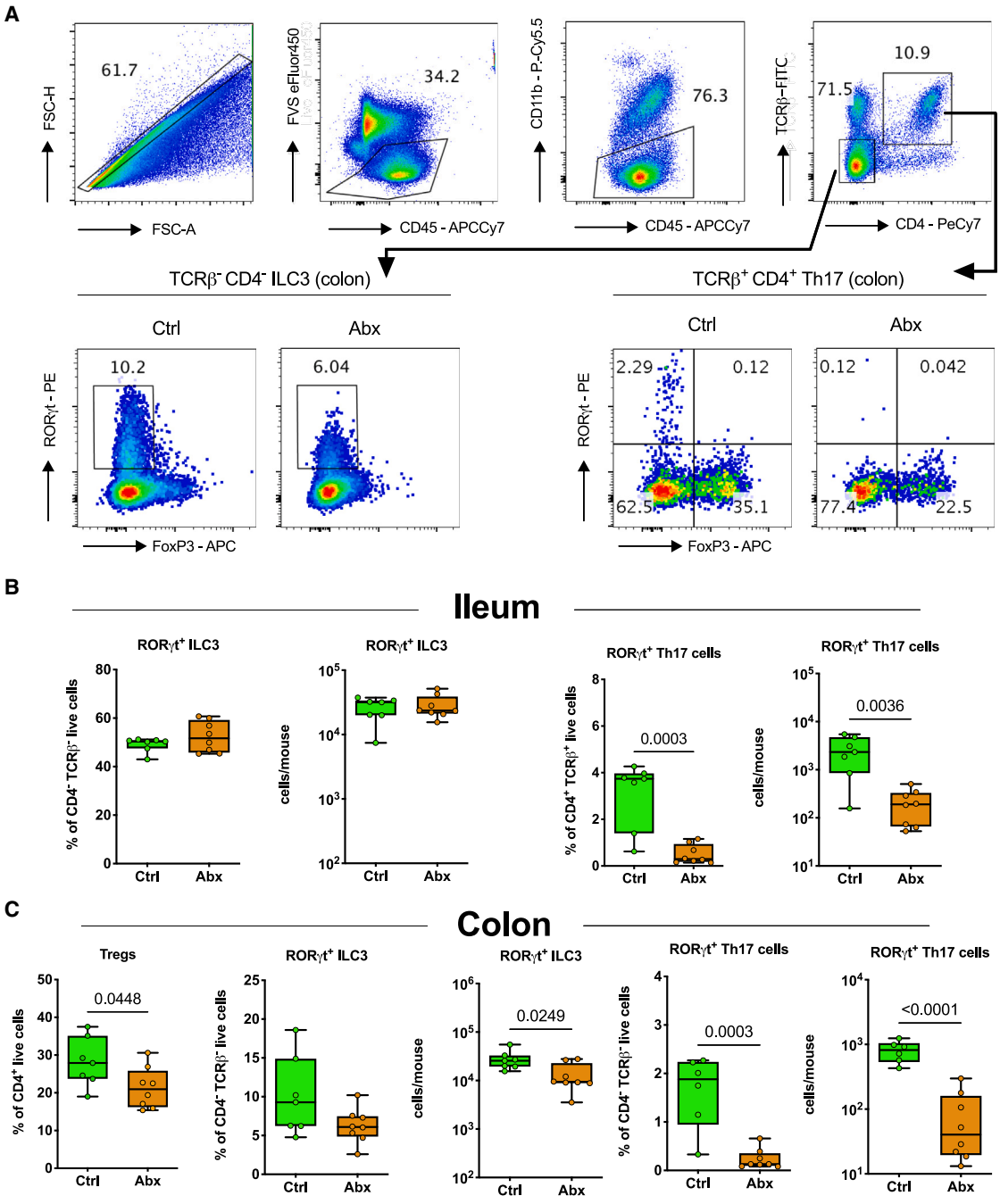

Figure 4. Effect of prenatal antibiotics on intestinal lymphoid cells

(A) Gating strategy used to identify ILC3 (CD45+ CD11b− CD4− TCRβ− FoxP3− RORγt+) and Th17 (CD45+ CD11b− CD4+ TCRβ+ FoxP3− RORγt+) cells in the lamina propria of ileum and colon.

(B) Proportion and absolute number of ILC3 and Th17 cells in the lamina propria of the ileum at 28 days of life, without (Ctrl) or with (Abx) previous Abx treatment of the mother during the last week of pregnancy. % are calculated on CD4− TCRβ− live cells for ILC3 and on CD4+ TCRγ+ live cells for Th17 cells.

(C) Same as (B) but for colonic lamina propria, including the percentage of Treg cells. $n = 7–8$ mice per group, representative of 2 independent experiments. For (B) and (C), p values calculated by t test. For (B) and (C), whiskers indicate minimum and maximum values.

established an experimental model of cross-fostering pups as soon as possible after birth, so that untreated ("Ctrl") mothers would breastfeed pups exposed *in utero* to Abx and vice versa (Figure 5A). At P7, the key factor influencing the amount of fecal SIgA was clearly breastfeeding: indeed, non-crossed pups replicated the pattern of fecal IgA highlighted previously, with pups born and breastfed by Abx-treated mothers having significantly less fecal IgA compared with the control group (Figure 5B). After

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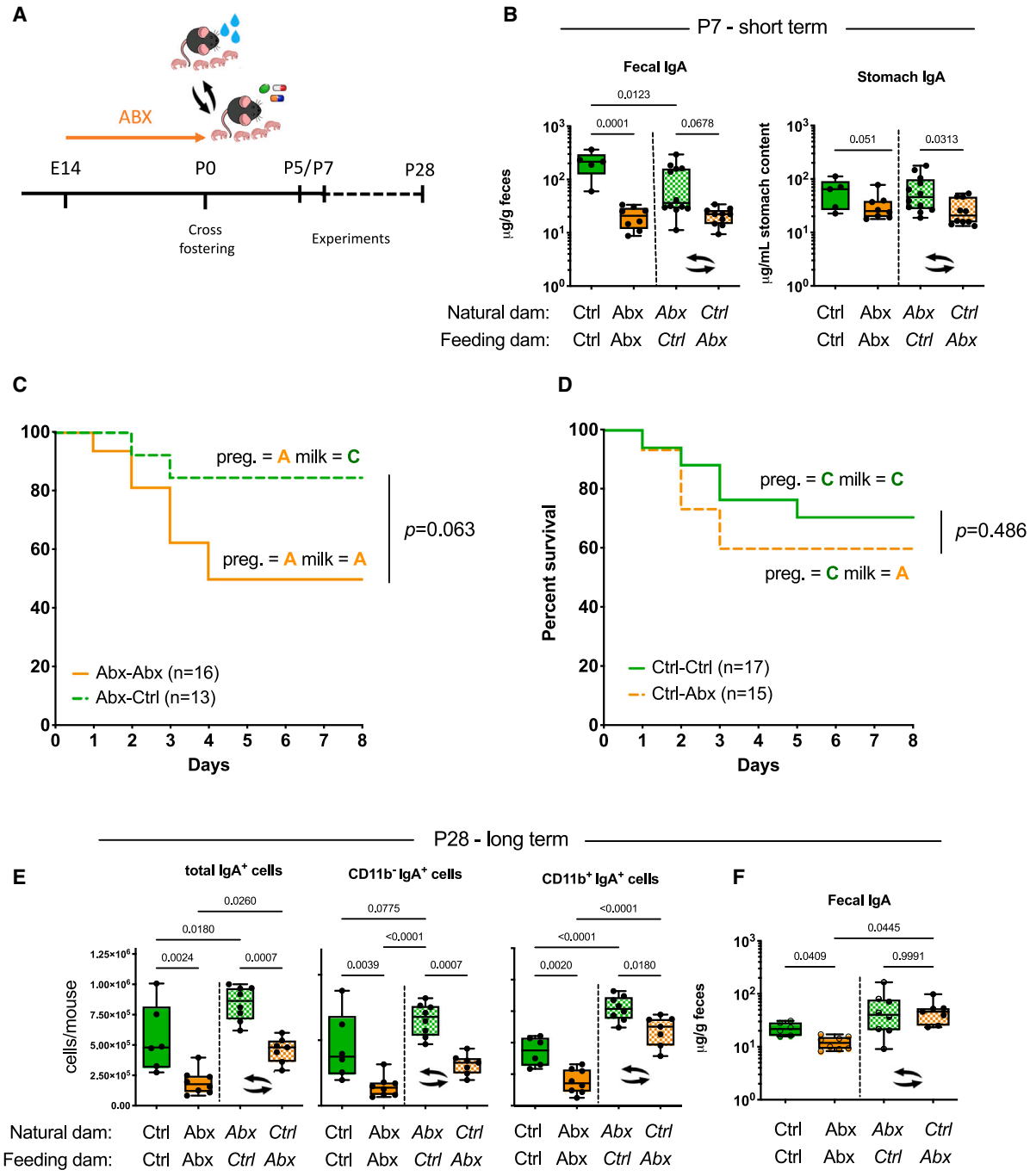

Figure 5. Fostering mother phenotype largely determines the trajectories of neonatal intestinal IgA

(A) Experimental scheme. After prenatal Abx treatment, litters were cross fostered within 12 h from birth and experiments were conducted at P5–P7 or P28.

(B) Amount of fecal IgA and stomach IgA in pups of 4 different experimental groups. $n = 7–8$ mice per group, representative of at least 2 independent experiments. p values calculated by two-way ANOVA with Sidak correction for multiple comparisons.

(C) Survival curves of pups born to Abx-treated mothers after cross fostering and gavage at P4–P5 of 1.6×10^9 CFUs of *E. coli K1*.

(D) Survival curves of pups born to untreated mothers after cross fostering and gavage at P4–P5 of 1.6×10^9 CFUs of *E. coli K1*.

(E) Amount of fecal IgA in young adults of the 4 different experimental groups. $n = 7–8$ mice per group, representative of at least 2 independent experiments. p values calculated by two-way ANOVA with Sidak correction for multiple comparisons.

(F) Small intestine IgA+ plasma cells at P28 in young adults of the 4 different experimental groups. $n = 7–8$ mice per group, representative of at least 2 independent experiments. p values calculated by two-way ANOVA with Sidak correction for multiple comparisons. For (B), (E), and (F), whiskers indicate minimum and maximum values.

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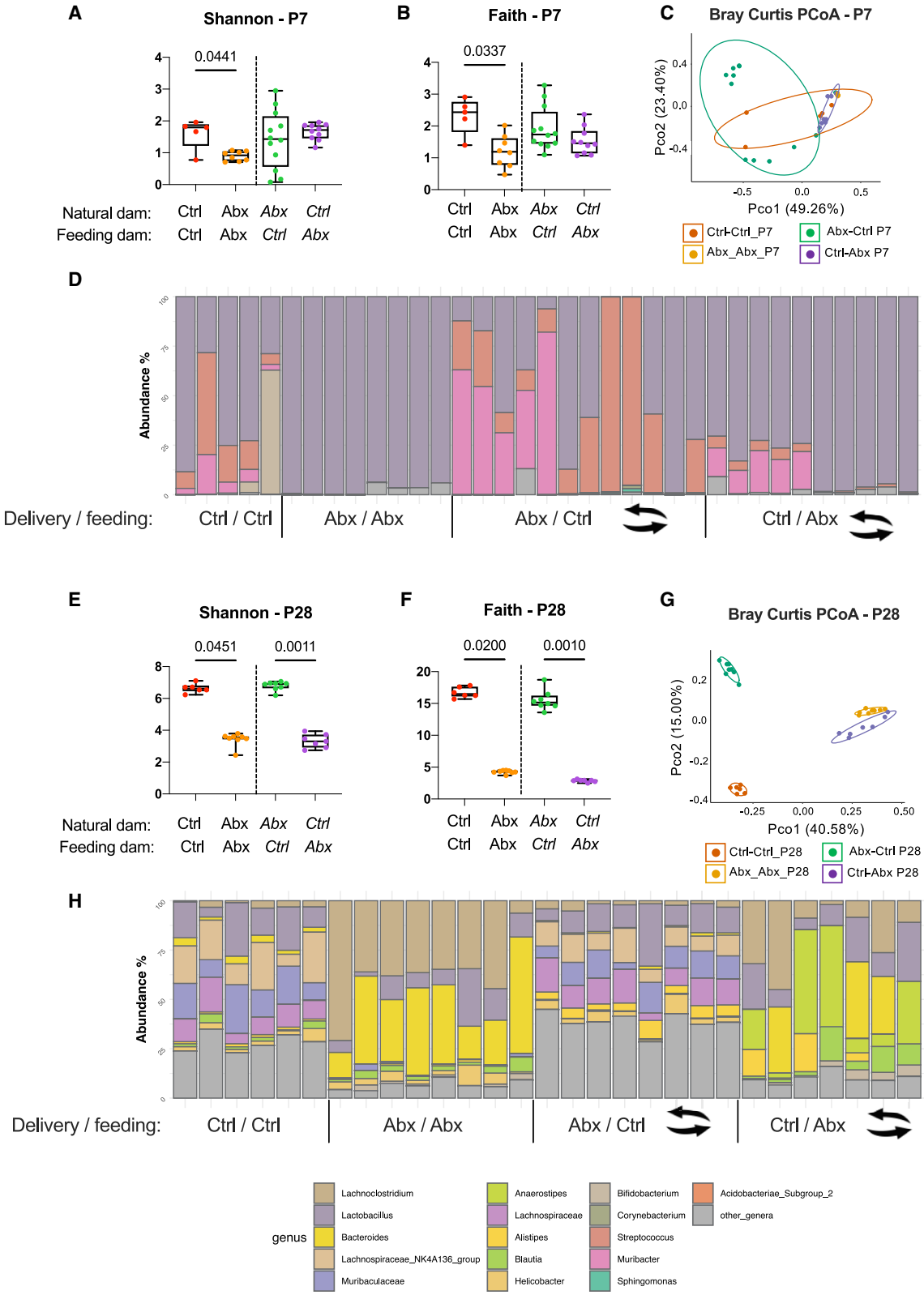

Figure 6. Prenatal antibiotics persistently alter neonatal fecal microbiota depending on breastfeeding mother

(A) α-diversity (Shannon index) of fecal microbiota at P7 in the 4 experimental groups of pups.

(B) α-diversity (Faith index) of fecal microbiota at P7 in the 4 experimental groups of pups.

(legend continued on next page)

cross-fostering (right part of each graph), pups born to control mothers but breastfed by Abx-treated foster dams had significantly lower fecal IgA compared with pups exposed to Abx *in utero* but breastfed by control mothers. These data were mirrored by the quantification of IgA in the stomach content of the same pups (Figure 5B, right). We then challenged cross fostered pups with *E. coli* K1 (Figures 5C and 5D). We observed that breastfeeding from control mothers could almost significantly rescue the phenotype of pups from prenatal Abx treatment (Figure 5C), while pups born to control mothers did not benefit from breastfeeding (Figure 5D), suggesting that untreated mothers could confer protection to the pups independently from breastfeeding. Whether this is related to the vertical transfer of some protective endogenous microbial strains during delivery remains to be established. Interestingly, breastfeeding by control mothers of pups born to Abx-treated mothers (green dashed line, in Figure 5C) led to a protective phenotype which seemed stronger than controls (green solid line, in Figure 5D), possibly due to the absence of competition of the pups microbiota for milk SIgA that leave them available to neutralize *E. coli* K1.

Regarding long-term outcomes (P28), we confirmed a reduced amount of IgA+ plasma cells (total, CD11b+ and CD11b−) of the ileum LP with a concomitant reduction in fecal IgA in young adult mice born to and raised by Abx-treated dams compared with those born to untreated ones (Figures 5E and 5F). After cross-fostering (right parts of each graph), mice born to Abx-treated mothers but breast fed by untreated foster mothers presented a proportion of IgA+ plasma cells in the LP of the ileum mirroring (or even higher than) that of young adults born to and raised by untreated mothers, while pups born to untreated mothers but raised by a previously Abx-treated mother presented significantly lower proportions of IgA+ plasma cells (total, CD11b+ and CD11b−) (Figure 5E). Interestingly, the absolute amount of total and CD11b+ IgA+ plasma cells were the lowest in pups born to and raised by Abx-treated mothers, while being born to an untreated mother contributed to higher amounts of IgA+ plasma cells even in the presence of a subsequent exposure to a maternal phenotype impacted by prenatal Abx. We speculate that since maternal IgAs are directed to her own microbiota,[8,33] a different emerging microbiota after Abx in the pups or that inherited by a different mother may lead to an increase of IgA-producing cells in the pups.

These data show that the phenotype of intestinal mucosa IgA+ plasma cells at P28 was partly shaped by breastfeeding, possibly explaining the protection of pups from control mothers breastfed by Abx-treated mothers.

At P28 both cross fostered groups of young adults presented fecal IgA levels similar to animals born to and raised by untreated mothers and higher than animals born to and raised by Abx-treated mothers (Figure 5F). Accordingly, but also due to *E. coli* strain K1 being predominantly a neonatal pathogen,[34] we showed no significant effect of prenatal Abx on survival to a late sepsis challenge (P28, Figure S3B). Interestingly, at an intermediate time point, the diminished survival of weanlings born to and raised by Abx-treated mothers was still appreciable (Figure S3A). Overall, these data highlight the importance of breastfeeding in shaping the neonatal secretion of IgA by the intestinal LP and suggest that the exposure to normal levels of breast milk IgA (and to a normal maternal microbiota) after birth may be a sufficient factor to almost re-establish the normal development of LP IgA-producing plasma cells.

Prenatal Abx induce long-lasting intestinal dysbiosis in neonates

Considered the tight, bidirectional relationship existing between luminal IgA and the gut microbiota, we analyzed the impact of prenatal Abx on fecal microbiota in our experimental groups. We selected two time points after birth: P7, when pups are exclusively breast fed, and P28, when weanlings are exclusively on a solid food diet. At P7, differences in α-diversity metrics (Shannon and Faith indexes) were moderate but significant between pups born to control or Abx-treated mothers (Figures 6A and 6B). This difference was lost when pups were either raised by or born to a control mother (Figures 6A and 6B). The Bray Curtis cluster analysis of compositional differences between experimental groups (Figure 6C) showed that pups born to and raised by Abx-treated mothers (orange dots) tended to cluster very close to each other, a sign of poor microbiota diversity. Conversely, pups born to or raised by untreated mothers presented a higher heterogeneity of microbial composition, with larger clusters. Relative abundances of genus-level taxonomies from 16s rRNA analysis (Figures 6D and S4) confirmed that pups born to or raised by Abx mothers presented a lower microbiota diversity, with a clear prevalence of Lactobacillus genus, while pups born to or raised by untreated mothers had a higher microbial diversity, being variably colonized by Lactobacillus, Muribacter, and Streptococcus genera. Bifidobacteria were present in the feces only when pups were born to and raised by control dams (Figure S4). To exclude that the phenotype was due to a significant transfer of maternal Abx directly into the breastmilk, we tested the levels of ampicillin, neomycin or erythromycin in the breast milk obtained from treated dams by mass spectrometry. As shown in Table S1, we could detect in breast milk only erythromycin and in just one sample, suggesting that the observed effect was not dependent on Abx transferred from the mothers via lactation.

At P28, the differences between experimental groups and the influence of breastfeeding became more striking. Regardless of

(C) β-diversity (Bray Curtis principal-component analysis) of fecal microbiota at P7 in the 4 experimental groups (indicated as mother/pups). For (A)–(C), $n = 5–12$ mice per group, representative of 2 independent experiments. p values calculated by Kruskal-Wallis test with Dunn's correction for multiple comparisons.
(D) Bar plots depicting the bacterial general of fecal pellets in the 4 experimental group (indicated as mother/pups) at P7.
(E) α-diversity (Shannon index) of fecal microbiota at P28 in the 4 experimental groups of pups.
(F) α-diversity (Faith index) of fecal microbiota at P28 in the 4 experimental groups of pups.
(G) β-diversity (Bray Curtis principal-component analysis) of fecal microbiota at P28 in the 4 experimental groups (indicated as mother/pups). For (E)–(G), $n = 5–12$ mice per group, representative of 2 independent experiments. p values calculated by Kruskal-Wallis test with Dunn's correction for multiple comparisons.
(H) Bar plots depicting the bacterial general of fecal pellets in the 4 experimental group (indicated as mother/pups) at P28. Legend applies to both (D) and (H). For (A), (B), (E), and (F), whiskers indicate minimum and maximum values.

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the natural mother, pups raised (and breastfed) by untreated mothers presented a significantly higher microbial diversity compared with pups fed by Abx-treated mothers (Figures 6E and 6F), and the treatment of the feeding mother determined the 40.58% of microbial diversity at Bray Curtis cluster analysis (Figure 6G). The composition of fecal microbiota was very similar between groups raised by untreated mothers, again independently from the natural mother of pups, and the same was true for pups raised by Abx-treated mothers (Figures 6H and S5). A contribution of coprophagy at this time point cannot be excluded. In particular, being fed by control mothers favored a higher complexity of the intestinal microbiome, with balanced populations of Lactobacillus, Muribaculaceae, Lachnospiraceae, and other genera, while breastfeeding by an Abx-treated mother caused a striking increase in Bacteroides, Lachnoclostridium, and Anaerostipes genera (this last predominantly in pups cross fostered and raised by an Abx-treated mother). We then sequenced the microbiota of excised mammary gland tissue at P7, P14, and P28 days after delivery to address its contribution in shaping neonatal fecal microbiota (Figure S6). Unexpectedly, we detected a significant increased Shannon index in Abx-treated dams at P7, which was later decreased at P14 compared with untreated dams. Shannon index quantifies the distribution and homogeneity of bacterial taxa, and these variations between 1 and 2 weeks after the interruption of Abx treatment might reflect different waves of mammary gland re-seeding by bacteria. The metrics results were mirrored by the relative abundance of bacterial genera at 16s rRNA analysis: the major visible impact of Abx treatment was highlighted at P14, when dams treated with Abx showed a clear predominance of a single genus, either Staphylococcus or Lactobacillus, and lower bacterial diversity (Shannon index), even though not statistically significant (Figure S6).

Overall, the trends identified by 16s rRNA sequencing of neonatal intestinal microbiota confirmed the absolute importance of breast milk components in shaping the content of gastrointestinal (GI) microbiota in the context of prenatal Abx. However, sequencing of the mammary gland microbiota did not reveal a clear or similar trend induced by prenatal Abx treatment, rather an increased microbial diversity in dams' mammary glands at P7. This suggests that the neonatal fecal microbiota composition in our experimental setting was unlikely to be inherited straight from breast milk bacteria, but was partially inherited during delivery, as already highlighted in humans,[35] and subsequently shaped by other factors such as breast milk IgA and/or possibly the ingestion of maternal feces (coprophagy).

DISCUSSION

Up to one-third of pregnant women receive at least one Abx prescription during pregnancy.[12,36,37] Nevertheless, the biological consequences of this exposure for fetuses and neonates have been only marginally investigated. Administration of antimicrobials is more common in late pregnancy, when permeability of the placental barrier is the highest and important milestones of fetal immune system development are ongoing.[38] Furthermore, when prenatal Abx are administered close to the delivery, their effect on mothers can extend into early post-natal life, as Abx-induced dysbiosis needs time to resolve.[39] Here, we discovered that prenatal Abx therapy increased the susceptibility to neonatal LOS caused by *E. coli* during the first week of life, reduced the transmission of breast milk SIgA to the suckling neonates, modulated the pups microbiota and significantly impaired the maturation of neonatal intestinal immune system, with effects persisting beyond the neonatal period. It is known that IgA+ plasma cells of the mammary gland originate from maternal MLNs, and migrate through the so called "entero-mammary pathway," driven by the chemokine gradient of the chemokine (C-C motif) ligand 28 (CCL28) expressed by the epithelial mammary tissue itself.[40,41] Considering our results, it is presumable that maternal intestinal dysbiosis causes a down regulation of LP and MLNs IgA-producing plasma cells in the pregnant mother, and a subsequent reduced migration toward the mammary gland during lactation. Conversely, we could show rather a higher α-diversity (Shannon index) of the mammary gland microbiota upon orally administered Abx, and no detectable levels of Abx in the breast milk of treated dams. Therefore, the phenotype we identified on neonatal gut, and even the modifications of neonatal fecal microbiota, seemed more correlated to the alterations in the functionality of the entero-mammary axis, and, possibly, to the vertical colonization of pups during vaginal delivery than to a direct perturbation of breast milk microbiota itself or to the transfer of persistently circulating Abx into breast milk.

Previously, it has been shown in a murine model that perinatal Abx can reduce circulating neutrophils in newborn mice, through a ILC3-G-CSF-mediated mechanism in the mucosa of the SI.[14] However, our experimental setting differed from most published models of perinatal dysbiosis[14,15,42,43]: as we aimed to investigate the effect of exposure to antimicrobials exclusively *in utero*, we exposed pregnant mothers to a short Abx course, with no direct exposure of suckling newborns to Abx. This, together with the absence of vancomycin in our experimental model (to better mimic antimicrobials administered to pregnant women), could account for the lack of major systemic immune shifts and significant alterations of the SI epithelial structure in pups. However, we observed a significantly increased susceptibility of pups born to Abx-treated dams to LOS induced by *E. coli*, a common gram-negative neonatal pathogen. The increased susceptibility correlated with higher bacterial translocation to distal organs, reduced amount of fecal SIgA, stomach IgA, and breast milk SIgA, and of bacterial coating by IgA themselves, including the specific coating of the pathogenic *E. coli* K1. SIgAs in the GI tract of newborns largely originate from breast milk[21,38] and represent a key protective factor against bacterial translocation,[44] neonatal sepsis,[45] and NEC.[11] This protection can be conferred even in the absence of antigen-specific IgA, i.e., by the broadly reacting, low-affinity "natural IgA."[8,46] As our females had never been exposed to *E. coli*, we speculate that the IgA we found in breast milk, as well as those coating *E. coli*, are natural, broadly specific IgA. The increased susceptibility to LOS and bacterial translocation after prenatal Abx treatment has never been reported previously, neither in humans nor in animal models. Reed and coll. reported a slight increase in LOS risk (OR 1.59; 95% confidence interval [CI] 0.84–2.99; $p = 0.15$) in a cohort of very preterm neonates exposed to prenatal Abx.[16] Nonetheless, the authors themselves pointed out how difficult it can be to clarify the effect of prenatal Abx in a real-life

scenario, where mothers are exposed to different molecules over different times.

We propose here that SIgA play a major role in protecting against LOS. Artificial restoration of breast milk IgA is cumbersome. Thus, we took advantage of an ATP-hydrolyzing enzyme, apyrase, that has been recently demonstrated to block the inhibitory effect of microbiota-derived ATP on Tfh cells and thereby amplifying the SIgA response.[23] Apyrase supplementation of Abx-treated dams promoted an increase of breast milk IgA and protected the pups from LOS. This finding together with the results of the cross-fostering experiments showing a rescue of the phenotype when pups born to Abx-treated mothers are breastfed from control mothers suggests a major role of SIgA in protecting the newborns from LOS. However, this protective role of SIgAs is observed only in pups born to Abx-treated dams, suggesting that other factors and most likely some microbiota members transferred by the mothers during the delivery may suffice to protect against LOS.

To ascertain whether prenatal Abx could also impact the progressive maturation of the neonatal intestinal immune function beyond the neonatal period, we analyzed the neonatal phenotype at a longer time point (4 weeks of age). We reasoned that the assembling gut microbiota, breast milk SIgA/solid food exposure, and the developing mucosal intestinal immune system formed a three-way, mutual connection.[47,48] In particular, the presence of both mucosal SIgA and of a normal intestinal microbiota are known to support the maturation of the intestinal mucosa immune system.[20,49]

In young adult mice born to and raised by Abx-treated mothers, we found a reduction in the absolute number of both CD11b+ and CD11b− IgA+ plasma cells. It has been recently shown that intestinal IgA+ CD11b+ plasma cells, compared with the CD11b− fraction, are more dependent on the presence of microbiome for their development, and on IL-10 signaling. However, when other cytokines induced by the microbiota, such as IL-5 or IL-6, are blocked, both fractions of plasma cells are reduced.[29] Our results are in line with these observations. Furthermore, as perturbations of the intestinal microbiota are known to have more profound effects if they occur early in life, we were not surprised that both CD11b+ and CD11b− fractions of IgA+ plasma cells were reduced at P28. Interestingly, we identified a preweaning interval (around P14) where the relative reduction of CD11b+ plasma cells of the SI LP was more pronounced in pups born to Abx-treated mothers, just before the CD11b− fraction of plasma cells became predominant in the LP. Other cellular populations of the SI LP were also affected by prenatal Abx treatment, such as DCs and mononuclear phagocytes (MNPs). In normal conditions, MNPs act locally in the SI LP as first line barrier against pathogens, while submucosal DCs are more mobile, carry antigens to the MLNs for T cell stimulation, or stimulate LP plasma cells to produce SIgA.[50,51] The development of both MNPs and DCs is favored by the presence of a normal intestinal microbiota, and hampered by Abx treatment[52]: here, we recapitulated this phenotype even without a direct exposure of pups to Abx. We also observed a reduction in colonic Tregs, and Th17 cells in both SI and LI after prenatal Abx treatment. Recently, it has been shown in a murine model that a prenatal, transient infection of a pregnant mother increases the amount of Th17 cells in the SI LP of the offspring in adult age, through epigenetic changes on fetal intestinal epithelial stem cells imposed by maternal IL-6.[30] We speculate that prenatal Abx therapy functionally represents a reflection of the effect reported by Lim and colleagues. However, even though at P28 the mucosal immune system of mice born from Abx-treated mothers was thoroughly impacted, mice were still resistant to LOS induced by *E. coli* K1. This may be due to the recovery of fecal SIgA or to a more developed mucosal barrier, which might contrast the pathogenicity of *E. coli K1* beyond the neonatal age.[34]

The cross-fostering experiments confirmed the predominant role of the fostering mother, i.e., of breast milk, in shaping both the composition of fecal microbiota and the abundance of luminal IgA, but it was evident primarily in pups born to Abx-treated dams. The effect of breastfeeding and of prenatal Abx on breast milk became even more prominent after the interruption of breastfeeding itself, in line with multiple previous observations highlighting the mutual role of microbiota and luminal IgA in shaping the development of intestinal immune system.[53–56]

In conclusion, we showed that prenatal Abx administered to pregnant mothers during the last stage of pregnancy profoundly increase the susceptibility of neonates to LOS, through the reduction of breastmilk SIgA and the modification of the newborn microbiota. Moreover, we highlighted that the breastfeeding window is key to maintain the homeostasis of gut immune system development later in life and further confirmed its protective role against neonatal sepsis. Being breastfed from untreated dams was even more important than vertical transmission of microbiota from mother to infant for the development of IgAs. Breastfeeding, with the contribute of breast milk SIgAs, was also fundamental for the correct diversification of the microbiota, and this is in line with our and others' reports that IgAs favor a positive feedback loop of IgA production and microbiota differentiation.[57,58]

The clinical implications of our data may be relevant: the results of a recent preliminary report by the group of Kathryn A. Knoop, not peer-reviewed yet, confirm lower IgA levels in the breast milk of women treated with prenatal Abx,[59] corroborating our findings on murine models. The confirmation of our findings in humans may prove important for different reasons: for example, the concentration of SIgA in breast milk is not a biological parameter routinely evaluated before the administration of milk to neonates, not even in the context of severe prematurity and human milk banking. The administration of Abx before delivery is a common situation in the context of prematurity, even more at earlier gestational ages, where the risk of subsequent neonatal LOS is the highest. If our data will be corroborated by evidence on human samples, the evaluation of SIgA concentration in breast milk of mothers who received Abx therapy before delivery might prove beneficial to select the most appropriate bank milk for each preterm neonate with the ultimate goal to reduce the incidence of harmful events such as LOS and NEC. In addition, as we recently found that a fermented formula encompassing *Lactobacillus-paracasei*-fermented milk favors the production of IgAs in neonates, as compared with a standard formula and similarly to that induced by breastfeeding,[60] one could consider the complementation with this type of formula when breastfeeding is not available or in a mixed regimen in case of mothers who have been treated with Abx prenatally. Finally, having observed a positive role of apyrase in driving

the production of SIgA, the supplementation with this protein to Abx-treated pregnant women may be an alternative to restore SIgA levels.

Limitations of the study

In our study, we tried to disentangle the role of maternal breastmilk IgA from that of the vertically transmitted microbiota. This is a very complex task as the two are strongly interconnected: when IgAs are knocked out the microbiota changes,[61,62] vice versa when the microbiota is affected by Abx, also IgAs are affected.[63] Thus, we decided to use apyrase and cross-fostering experiments, which we think are best suited to distinguish as much as possible between the two variables without interfering with each other. Our results suggest that breastmilk IgA is very important in protecting the pups from LOS, however, the microbiota transferred at birth can also overcome maternal IgA loss. We cannot exclude that in other models and with other microbial challenges the situation may be different and that also the breastmilk microbiota may participate in conferring resistance to enteropathogens, as recently shown in the context of complement (C1) deletion and *Citrobacter rodentium* infection.[64] In that case, complement in breastmilk controlled the composition of the microbiota even before reaching the gut through direct killing in particular of *Staphylococcus lentis*. Thus, other immune components such as complement may also be involved besides IgA in shaping the microbiota and protecting from neonatal bacterial challenges. Although we have used Abx and treatment schedule used in the clinics, we cannot anticipate that a similar protective effect is observed also in the human setting. However, the recent finding that breastmilk from Abx-treated mothers is reduced in IgA levels[59] suggests that a similar scenario may be present also in infants.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to Maria Rescigno (maria.rescigno@hunimed.eu).

Materials availability

The *E. coli mCherry K1* generated in this study will be made available on request, but we may require a payment and/or a completed materials transfer agreement if there is potential for commercial application. Additional information or inquiries should be sent to the lead contact (maria.rescigno@hunimed.eu).

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

C.P. ideated, conducted the experiments, and analyzed the data. C.C., M.L., A.S., T.R.J., P.B., G.F., S.C., B.D.P.C., L.M., A.B., and S.G. contributed to experiments and data analysis. D.B. and M.M. performed bioinformatic analyses. G.P., L.P., F.M., F.G., G.M., and M.R. provided scientific discussion. G.P., F.G., and M.R. helped in designing experiments. C.P., L.P., F.M., and M.R. conceived the study and interpreted the data. C.P. and M.R. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
anti-mouse PV-1	BD Biosciences	Cat #553849; RRID:AB_395086
anti-mouse CD34 Alexa Fluor 488	eBioscience	Cat # 53-0341-82; RRID:AB_2866440
anti-mouse CD34 Alexa Fluor 647	BD Biosciences	Cat # 560230; RRID:AB_1645200
anti-mouse CD16/CD32 unconjugated	eBioscience	Cat # 14-0161-82; RRID:AB_468899
anti-mouse CD45.1 FITC	Biolegend	Cat # 110706; RRID:AB_1727495
anti-mouse CD45.1 APC-Cy7	Biolegend	Cat # 110716; RRID:AB_1727495
anti-mouse CD45.2 FITC	Invitrogen	Cat # 11045485; RRID:AB_394528
anti-mouse CD45.2 APC-Cy7	Biolegend	Cat # 109824; RRID:AB_394528
anti-mouse CD3 PeCy7	BD Biosciences	Cat # 552774; RRID:AB_893318
anti-mouse CD11b BV421	BD Biosciences	Cat # 562605; RRID:AB_396784
anti-mouse CD11b PeCy7	BD Biosciences	Cat # 552850; RRID:AB_396784
anti-mouse Ly6C BV605	BD Biosciences	Cat # 563011; RRID:AB_1727554
anti-mouse Ly6G BV510	Biolegend	Cat # 127633; RRID:AB_2562937
Anti-mouse CD3e PeCy7	BD Biosciences	Cat # 552774; RRID:AB_394460
Anti-mouse CD4 PE	BD Biosciences	Cat # 553048; RRID:AB_394584
Anti-mouse CD8 APC	BD Biosciences	Cat # 553035; RRID:AB_398527
Anti-mouse TCRβ FITC	BD Biosciences	Cat # 553171; RRID:AB_394683
Anti-mouse FoxP3 APC	eBioscience	Cat #17-5773-82; RRID::AB_469457
Anti-mouse RORγτ PE	eBioscience	Cat # 12-6988-82; RRID:AB_1834470
Anti-mouse CD103 PE	Biolegend	Cat # 121406; RRID:AB_1133989
Anti-mouse CX3CR1 AF647	Biolegend	Cat # 149004; RRID:AB_2564273
Anti-mouse MHC-II BV421	BD Biosciences	Cat # 562564; RRID:AB_2716857
Anti-mouse CD138 PerCP-Cy5.5	Biolegend	Cat # 142509; RRID:AB_2561600
Anti-mouse CD11c PerCP-Cy5.5	eBioscience	Cat # 35011482; RRID:AB_469709
Fixable Viability Dye eFluor® 780	eBioscience	Cat # 65-0865-14;
Bacterial and Virus Strains		
E. coli serotype O18:K1:H7	Leibniz Institute DSMZ	Cat # 0719
E. coli mCherry O18:K1:H7	This paper	N/A
Chemicals, Peptides, and Recombinant Proteins		
neomycin sulfate	GoldBio	Cat # N-620-100
ampicillin sodium	GoldBio	Cat # A-301-100
Erythromycin	NeoBiotech	Cat # NB-42-01217-100G
4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI)	Invitrogen	Cat # D1306
VECTASHIELD Mounting Medium	Vector Laboratories	Cat # H-1000
FITC-Dextran 4 kDa MW	Sigma-Aldrich	Cat # 46944-500MG-F
Collagenase D	Roche	Cat # 11088866001
Poly(vinylpolypyrrolidone) (PVPP)	Sigma-Aldrich	Cat # 77627-25G
MRS broth with Tween 80	Biolife italiana	Cat # 4017292
Luria Bertani (LB) Broth	BD Difco	Cat #244620
Critical Commercial Assays		
Quick-RNA MiniPrep	Zymo Research	Cat # ZYR1055
ImProm-II Reverse Transcriptase kit	Promega	Cat # A3803
Fast Sybr Green PCR kit	Applied Biosystems	Cat # 4385614

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
G'NOME DNA isolation kit	MPBiomedicals	Cat # 112010600
Experimental Models: Organisms/Strains		
Mouse: C57BL/6JOlaHsd	Envigo	Cat # 057;
Mouse: C57BL/6	Charles River	Cat # C57BL/6NCrl
Oligonucleotides		
Reg3γ QuantiTect Primer Assay	QIAGEN	Cat No 249900; GeneGlobe ID QT00147455
Def-α5 QuantiTect Primer Assay	QIAGEN	Cat No 249900; GeneGlobe ID QT00280315
Mucin-2 QuantiTect Primer Assay	QIAGEN	Cat No 249900; GeneGlobe ID QT01060773
Occludin QuantiTect Primer Assay	QIAGEN	Cat No 249900; GeneGlobe ID QT00111055
ZO-1 QuantiTect Primer Assay	QIAGEN	Cat No 249900; GeneGlobe ID QT00493899
PLVAP QuantiTect Primer Assay	QIAGEN	Cat No 249900; GeneGlobe ID QT00290584
Recombinant DNA		
Plasmid: pONmCherry	Addgene	Addgene plasmid #84821; RRID:Addgene_84821
Software and Algorithms		
Fiji	ImageJ	http://fiji.sc; RRID:SCR_002285
FlowJo v10.5.3	TreeStar	https://www.flowjo.com/solutions/flowjo; RRID: SCR_008520
Trimmomatic v0.39	Bolger et al., 2014[65]	http://www.usadellab.org/cms/?page=trimmomatic; RRID:SCR_011848
cutadapt v1.18	Martin, 2011[66]	https://cutadapt.readthedocs.io/en/stable/index.html; RRID:SCR_011841
Qiime2 v2019.7	Bolyen et al., 2019[67]	https://qiime2.org/; RRID:SCR_018074
Prism	GraphPad	http://www.graphpad.com/; RRID: SCR_002798
Other		
Columbia agar plates (5% sheep blood)	Oxoid	Cat # PB5039A
0,1 mm Zirconia/Silica Beads	BioSpec Products	Cat # 11079101z
Count Bright Absolute Counting Beads	Invitrogen	Cat # C36950

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Mouse models

Female C57BL/6JOlaHsd mice, 8-12 weeks old, were purchased from Envigo RMS (Udine, Italy). Male C57Bl/6 mice for breeding, 8-12 weeks old, were purchased from Charles River Laboratories (Calco, Italy). Mice were housed under specific-pathogen free (SPF) conditions at Humanitas Clinical and Research Center (Rozzano, Milan, Italy) and at Institute for Research in Biomedicine (Bellinzona, Switzerland), with food and water ad libitum and a 12-hours dark-light cycle. Females were co-housed in cages of 5 animals, males were single housed. All animal experiments were performed under protocols (n. 567/2019-PR in Rozzano and 34591 TI/02/2022/2024 in Bellinzona). approved by the Italian Ministry of Health (Italy) and the Cantonal Veterinary (Switzerland), and consistent with national (D.L. N. 26, G.U. March 4, 2014 in Italy, Swiss Federal Veterinary Office guidelines in Switzerland) and international laws and policies (EEC Council Directive 2010/63/EU). Breedings were established with a 1:1 or 1:2 scheme in the late afternoon, after synchronization of female estrous cycles, and the presence of vaginal plugs was verified in the early morning. In the presence of plug, gestational age was counted starting at 0.5 days. To ensure homogeneous microbiome before experimental treatment, synchronized pregnant females were co-housed until the beginning of antibiotic treatment, when they were single housed.

Antibiotic treatment

At gestational age E13.5, pregnant females were randomly assigned to receive either sterile drinking water or a cocktail of clinically relevant antibiotics composed of Ampicillin (1g/L), Neomycin (1g/L) and Erythromycin (0.1 g/L), dissolved in 200 mL of sterile drinking water. Antibiotics and dosage were chosen to mirror the exposure to antimicrobials of pregnant women in case of pPROM and of neonates in the peripartum period. Antibiotics were replaced every other day until E18.5, when they were removed to avoid direct exposure of pups to the treatment after birth. After birth, pups were left with their own mother or, in case of cross-fostering experiments, pooled between the same treatment groups and re-assigned to a different mother, with homogeneous litter sizes. In any experiment, in case of litter smaller than 4 pups, the whole litter was not used. In case of litters exceeding 8 pups, pups were

sacrificed to ensure homogeneous litter size. Pups remained with the fostering mother throughout the entire experiments, except for separation during short periods (maximum: 4 hours) of fasting when necessary for experimental procedures.

Apyrase treatment

At gestational age E9.5 until day P4 after delivery, pregnant females were randomly assigned to receive either PBS or recombinant Apyrase (40 μg/mouse) administered through oral gavage once a day in a total volume of 100 μL PBS/mouse. After birth, pups were left with their own mother. Pups remained with the fostering mother throughout the entire experiments.

Sepsis model

Escherichia coli serotype O18:K1:H7 (DSMZ #10719) was grown (37°C, 180 r.p.m.) in Luria Bertani (LB) broth overnight, and the culture was restarted in early morning to log-phase growth. To mimic *E. coli* late-onset neonatal sepsis due to bacterial translocation from the intestinal lumen, neonatal mice (postnatal day 4-5, "P4-P5") were gavaged with *E. coli* (1 × 109 CFU g−1, approximately 3 × 109 CFU/mouse at P4-P5, in 50 μL of 1X PBS). To generate survival curves, pups were inspected every 8-12 hours for the following 10 days and sacrificed if moribund according to standardized procedures.[68] To evaluate bacterial burden in distant organs, as well as cytokines/chemokines serum levels, infected pups were sacrificed by decapitation 24 or 48 hours after the infection. Spleen, liver, mesenteric lymph nodes (MLNs) and fecal pellets were collected under sterile conditions, submersed in 70% alcohol for 30 seconds to kill superficial bacteria (except feces), and homogenized in sterile phosphate buffer saline (PBS) (for liver and MLNs, a preliminary step of digestion with collagenase D at 37°C, for 30 minutes on rotating support was performed). Serial dilutions of organs homogenates (or feces, when necessary) were plate on *E. coli*-specific agar plates (Chromoselect, Millipore), where *E. coli* colonies assume a dark blue color. Immediately before plating, an equal volume of sodium deoxycholate solution was added to the organ homogenate to ensure mammalian cell disruption. Plates were incubated at 37°C overnight.

METHOD DETAILS

Construction of E. coli mCherry K1

E. coli K1 was transformed by electroporation using pONmCherry plasmid, a gift from Howard Shuman (Addgene plasmid # 84821).[69] One hundred ml of exponential phase cultures *E. coli K1* (0D600= 0.6) were cooled on ice for 15 min and then harvested by centrifugation at 4000 g for 10 min at 4°C. After washing the pellet twice with 100 ml ice-cold distilled water, cells were re-suspended in 1 ml ice-cold 10% glycerol, ready for electroporation. For each transformation, 40 μl cells were mixed with around 100 ng of plasmid DNA, transferred to a chilled 2-mm gap cuvette (Bio-Rad, USA) and incubated on ice for 10 min. The mixture was then electroporated at 2,500 V, 200 Ω and 25 μF, using a Gene Pulser XcellTM electroporator (Bio-Rad, Munchen, Germany). Then the electroporated cells were transferred in 500 μl of fresh SOB medium supplemented with 10 μl/ml MgCl2 and 20 μl/ml Glucose allowing them to recover by incubation at 37°C for 1 h. Cells (100 μl) were then plated onto selective LB agar plates with 34 μg/ml chloramphenicol to recover transformants. Expression of the mCherry gene did not alter the growth rate of the bacterium.

Recombinant Apyrase purification

The phoN2 gene, encoding periplasmic ATP-diphosphohydrolase (apyrase) of Shigella flexneri, was cloned in the pET21a vector (Novagen 69740) with a C-terminal His-Tag sequence. Transformed Rosetta (DE3)pLysS cells (Novagen 70956) with pET21a vector were grown on LB-agar (Sigma L2897) plates with 34 μg/ml chloramphenicol and 100 μg/ml ampicillin and single colony amplified in LB medium (Sigma L3032) with the same antibiotics. Apyrase expression was induced with 0.5 mM isopropyl-1-thio-β-d-galactopyranoside (IPTG, Sigma I6758). For apyrase purification, the culture was harvested and the resulting cell pellet, after a washing step in 10 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, was resuspended in 50 mM Tris-HCl, pH 7.5, and snap frozen in liquid nitrogen. After thawing and addition of 300 mM NaCl and 40 mM imidazole (Sigma I202), the cell suspension was sonicated and cleared by centrifugation at 15'000 rpm for 45 min at 4 °C. The lysate was then placed on a Pierce™ Disposable Column (Thermo Scientific) containing 4 ml of Ni-NTA agarose beads (Qiagen) and passed by gravity flow. After two washing steps of the column with lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 40 mM imidazole), the bound protein was eluted in elution buffer containing 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 300 mM imidazole. Purity and amount of recovered apyrase were assessed by SDS-PAGE gel (Thermo Scientific) and staining with Imperial Protein Staining (Thermo Scientific), respectively. Fractions containing the enzyme were pooled and concentrated to 5 ml using a 10-kDa MWCO spin concentrator (Amicon ultra-15, Millipore) and then loaded onto a Hi load s-75 size exclusion column (Cytiva) equilibrated in phosphate-buffered saline (PBS, Sigma D8537). The peak fractions were pooled and concentrated using a spin concentrator. The purified apyrase was filtered using a 0.22 μm Millex®-GP filter (Millipore) and stored in aliquots at 4°C.

Immunoglobulin's quantification

Feces and stomach content of the pups (as a proxy of maternal breastmilk) were weighted and frozen in dry ice immediately after collection. Serum was collected after centrifugation (5000 x g, 5 minutes) of clotted blood. To quantify the levels of IgA and IgG, defrost fecal pellets and stomach contents were dissolved in 200 μL PBS containing 0.1 mg/ml soybean trypsin inhibitor. Complete homogenization of samples was achieved with agitation in Tissue Lyser (Qiagen) for 30 seconds at 30 Hz, after the addition of 1.4 mm ceramic spheres (MP Biomedicals) to the tubes. Debris were removed by centrifugation (50 x g. 10 minutes, 4°C). 96-well ELISA

plates (NUNC Maxisorp) were coated overnight with 150 ng/mL of goat anti-mouse IgA antibody (Southern biotech 1040-01) in 1X PBS. IgA and IgG concentration in serial dilutions of the samples were detected with horseradish peroxidase (HRP)-conjugated anti-mouse IgA (SouthernBiotech). HRP activity was detected with TMB substrate (BD Biosciences), the reaction was stopped with 2M Sulfuric Acid, and absorbance was measured at 450 nm.

Cellular isolation

To isolate leukocytes from the LP of SI and colon, the intestine was removed from the mouse, opened longitudinally, and placed in ice-cold PBS. Up to P14, the whole SI and colon were processed, to ensure the recovery of enough cells. From day of life 15, the whole colon and approximately 15 centimeters of distal SI were processed. Residual fat and mesenteric lymph nodes were removed. The intestines were sectioned into 1 cm segments and washed twice for 20 minutes in 8 ml of PBS without calcium and magnesium, with 2 mM EDTA and 5% FBS with shaking at 37°C, 240 RPM, with horizontal tubes, to detach epithelial cells. After each wash, content of tubes was poured onto a metal strainer, and tissue pieces recovered with forceps. Supernatant containing epithelial cells was discarded. Residual tissue was then digested in 8 ml of PBS with calcium and magnesium, containing 5% FBS, 0.5 mg/ml collagenase type VIII (Sigma) and 10 U/ml DNase I (Roche), with shaking at 37°C for 25-30 min (small intestine) or 30 min (colon). The resulting cell suspension was passed through a cell strainer (70 μm) and washed with 8 ml of FACS buffer (PBS, 2% FBS, 2mM EDTA). Cells were centrifuged (450 x g., 5 min, 4°C) and resuspended in 200 μl of FACS buffer. Both adult and neonatal intestines were processed individually.

Livers and lymph nodes were digested in PBS (2% FBS) containing collagenase type D (1 mg/ml, Sigma) at 37°C for 30 min. Cellular suspensions were also passed through a cell strainer (70 μm) and washed with FACS buffer. Spleens were directly crashed on a cell strainer with a syringe plunger. To isolate circulating leukocytes, blood was collected after euthanasia by cardiac puncture or by decapitation (for mice younger than 10 days old) in heparinized tubes (3μL of 5000U/mL solution per tube). After centrifugation and plasma collection, pellets were resuspended in 1 mL of NH_4Cl buffer (8.34 g ammonium chloride, 0.037 g EDTA and 1 g sodium hydrogen carbonate/L, pH 7.2) for 1-2 minutes at room temperature to obtain the lysis of red blood cells (RBCs). After two washes in FACS buffer, cells were finally resuspended in 200 μl of FACS buffer. Erythrocytes in neonatal splenic suspensions were lysed for 30 seconds at room temperature as well.

Flow cytometry

After processing, cells were blocked with anti-CD16/32 antibody in FACS buffer for 20 minutes at 4°C, then surface-labeled with the antibodies indicated in the key resources table for 30 minutes at 4°C in FACS buffer. Dead cells were excluded by using Fixable Viability Dye eFluor00E2 510 or 780 (564406 and 565388 respectively, BD Biosciences, 1:1000), in protein-free PBS. For the detection of intracellular cytokines, IgA, and nuclear factors (such as Foxp3 and RORγt), cells were then permeabilized using an intracellular staining kit (BD, #554714 for cytoplasmic proteins/IgA, or eBioscience, #00-5523-00 for nuclear factors) and stained with the appropriate amount of intracellular antibody resuspended in permeabilization buffer, for 1h (nuclear factors) or 30 minutes (IgA and cytokines) at RT. Cells were fixed in 1% paraformaldehyde for 10 minutes before storage at 4°C. Flow cytometry was performed on FACSCanto II and Fortessa (BD Biosciences) platforms, and results were analyzed using FlowJo software (Tree Star, version 10.5.3). Every gating analysis started from a gate on single, live, CD45+ cells. Absolutes numbers were calculated using Count Bright Absolute Counting Beads (Invitrogen #C36950).

Staining of fecal bacteria for IgA

To identify IgA+ bacteria in fecal samples, a modified version of the protocol by Palm and coll. was applied.[70] Briefly, frozen fecal pellets were resuspended in 200 μl of FACS buffer with 1.4 mm ceramic beads (MP Biomedicals) and homogenized by agitation on a Tissue Lyser (Qiagen) for 30 seconds at 30 Hz. Then, slow centrifugation (50 x g. for 10 minutes, 4°C) was applied to remove large particles, leaving bacteria in suspension. Supernatants were collected and spun down at 4000g x 10 minutes (4°C) to retrieve bacterial pellets, that were then resuspended in FACS buffer, blocked with anti-CD16/32 solution in FACS buffer (20 minutes, 4°C) and stained with anti-IgA PE-conjugated antibody (clone mA-6E1, 1:100) for 30 minutes at 4°C. Before acquisition of data, a suspension of unlabeled Gram+ (*Lactobacillus paracasei*) and Gram- (*E. coli K1*) bacteria was used to set the gates for identification of bacteria.

Immunofluorescence

After animal euthanasia, pieces murine tissues were fixed overnight in "PLP" buffer (1% paraformaldehyde, L-Lysine 0.2M pH 7.4 and 1.56 mg/mL NaIO4) at 4°C. Then they were washed, re-hydrated with 20% sucrose for at least 4 hours at 4°C and included in optimal cutting temperature (OCT) gel (Sakura), frozen at -80°C. Ten-mm cryosections were cut, rehydrated, blocked and permeabilized with 0.1M Tris- HCl pH 7.4, 2% FBS, 0.3% Triton X-100 for 10 minutes at RT. Then sections were stained with the following antibodies: goat anti-Mouse IgA-UNLB (1:400, Southern Biotech #1040-01), anti-mouse PV-1 (clone MECA-32, 1:100 BD Pharmigen), anti-mouse CD34-Alexa Fluor 488 (clone RAM34, 1:100 eBioscience) or anti-mouse CD34-Alexa Fluor 647 (clone RAM34, 1:50 eBioscience), anti-mouse ZO1 Alexa Fluor 488 (clone ZO1-1A12, 1:50 Invitrogen), anti-mouse Mucin-2 (MUC2, clone F-2, 1:200, Santa Cruz). Primary and conjugated antibodies were incubated o/n at 4°C. Slices were then washed and incubated with the appropriate fluorophore-conjugated secondary antibody, for 2 hours at room temperature. Before imaging, nuclei were counterstained with (DAPI), and cytoskeleton with phalloidin if necessary. Confocal microscopy was performed on a Leica TCS SP5/SP8

laser confocal scanner mounted on a Leica DMI6000B inverted microscope equipped with motorized stage. Violet (405nm laser diode), yellow (561nm laser diode) and red (633nm laser diode) laser lines were used for excitation. All images were acquired with an HCX PL APO 40X oil immersion objective. Leica LAS AF was used for acquisitions. Images were then analyzed with FIJI open-source software.

RT-qPCR assay

Murine frozen tissues (ileum, colon) were homogenized using Tissue Lyser (Qiagen). Total RNA was purified using Quick-RNA MiniPrep (Zymo Research), according to manufacturer protocol. At the last protocol step, mRNA was eluted in 30 μL of pure H_2O. cDNA synthesis was performed using ImProm-II Reverse Transcriptase kit (Promega) following manufacturer's instruction and random primers (0,5 μg/ml, Invitrogen). Real-time PCR reactions were carried out using the Fast Sybr Green PCR kit (QuantiStudio 7 Flex RealTime PCR, Applied Biosystems). The relative expression levels were calculated by the delta-delta CT method ($2^{-\Delta\Delta Ct}$), after normalization to the average of Rpl32 or 18s rRNA level, for murine gene expression and amplification of bacterial genes respectively.

Stool bacterial load was quantified by analyzing the level of 16s rRNA gene expression, normalized to the average of 18S rRNA.

Quantification of antibiotic molecules in breast milk

Erythromycin, Ampicillin and Neomycin content in mice milk was quantified by LC-MS/MS detection on a TSQ Altis™ Triple Quadrupole Mass Spectrometer (Thermo Scientific) operating in positive ion mode monitoring the transitions m/z 734.55>558.48 (quantitation ion) and m/z 734.55>576.47 (confirmation ion) for Erythromycin, m/z 350.11>360.12 (quantitation ion) and m/z 350.11>192.19 (confirmation ion) for Ampicillin, m/z 308.25>293.20 (quantitation ion) and m/z 308.25>455.29 (confirmation ion) for Neomycin, and m/z 370.16>353.20 (quantitation ion) for D4-Amoxicillin stable deuterium standard that was used as internal standard. Positive ion voltage was set at 4000 V, sheath gas, aux gas and sweep gas were set at 50, 10 and 2.5 respectively. Ion transfer tube temperature was 325°C and vaporizer temperature was 350°C. Chromatographic separation was achieved on a Kinetex™ EVO C18 column (110A, 2.1 x 100 mm, 2.6 μm particle size; Phenomenex Inc., Torrance, CA, USA) at 35 °C at 0.2 ml/min using H2O plus Formic acid (HCOOH) 0.1% (mobile phase A) and Acetonitrile (CH3CN) plus Formic acid 0.1% (mobile phase B) under gradient conditions as follows: 5–90% B (0–5 min), 90% B (5–6 min), 90–5% B (6–6.1 min), 5% B (6.1-15 min). Milk samples were added with Acetonitrile to precipitate protein. After centrifugation at 15000xg, the supernatant and pellet were collected separately. Supernatant was evaporated to dryness under N2 flux to measure Erythromycin and Ampicillin. The extracts were reconstituted in 100μl of mobile phase B and 10 μl were injected into the HPLC system. The pellet was added with 100μl of mobile phase A, sonicated for 10 minutes, and 10 μl were injected into the HPLC system. Study samples were assayed together with a five-point calibration curve in untreated milk at concentrations ranging from 1 to 1000 ng/ml for Erythromycin and Ampicillin and from 0.25 to 10 μg/ml for Neomycin. The limit of quantification (LOQ) was 1 ng/ml for Erythromycin and Ampicillin and 0.25 μg/ml for Neomycin.

FITC-dextran assay

Before the assay, pups were starved for 4 hours separating them from the dam, adult mice were starved over-night. Then, both pups and adult mice were orally administered by gavage 400 mg/Kg of FITC-Dextran (4 kDa; Sigma-Aldrich, # 46944-500MG-F). Blood was collected from the tail (adult mice) or by terminal decapitation (pups) after four hours, and the concentration of FITC-Dextran in serum samples was measured as fluorescence intensity after dilution in PBS (Clariostar Plus Microplate Reader; BMG Labtech).

Bacterial DNA extraction and quality control from feces and mammary glands

DNA from fecal pellets was extracted with G'NOME DNA isolation kit (MP) following a published protocol.[71] Briefly, fecal pellets, frozen at -80°C, were homogenized in 550 μl Cell Suspension Solution (G'NOME DNA Kit). After addition of 50 μl RNase Mix (G'NOME DNA Kit) and 100 μl Cell Lysis/Denaturing Solution (G'NOME DNA Kit) samples were incubated at 55°C for 30 minutes. After adding 25 μl Protease Mix (G'NOME DNA Kit) samples were incubated for further 2 hours at 55°C. Samples then underwent mechanical disruption of bacterial cells with 0,1 mm zirconia/silica beads (BioSpec #11070101z) in FastPrep-24™ homogenizer (MP Biomedicals). Lysates were retrieved. Beads were washed two times with 400 μl of TENP buffer (50 mM Tris pH 8, 20 mM EDTA pH 8, 100 mM NaCl, 1% PVPP). Supernatants were pooled with the original lysate and precipitated with isopropanol. DNA pellets were resuspended in 400 μl water and incubated with 100 μl of Salt Out Mixture (G'NOME DNA Kit) to remove impurities. Samples were then precipitated in 100% ethanol and DNA pellets washed with 70% ethanol. DNA pellets were dried and resuspended in molecular H_2O.

Bacterial DNA extraction from murine mammary glands was performed with DNeasy PowerSoil Pro Kit (QIAGEN) following the manufacture's protocol. First, mammary glands were excised with sterile surgical instruments from dermal flaps after sacrifice, and immediately frozen at -80°C. Each thawed gland was added to 800 μl CD1 (QIAGEN) suspension solution and homogenized with a tissue homogenizer (TissueRuptor, QIAGEN). Homogenized tissues were added to PowerBead Pro Tubes (QIAGEN), vortex briefly to mix and mounted on a TissueLyser (QIAGEN) to shake for 10 min at speed 25 Hz. Samples were subsequently processed according to the manufacture's protocol. The final elution volume of DNA was 50 μL. After extraction, DNA concentrations were measured by NanoDrop™ 8000 Spectrophotometer (Thermo Fisher Scientific). DNA samples were stored at − 20°C until sequencing. DNA quality control was performed with the Agilent 4200 Tape Station system using the High Sensitivity DNA ScreenTape analysis kit (Agilent, Santa Clara, CA, USA), only DNAs having a DIN>6.5 were used for library preparation.

Analysis of the microbiota composition by 16S rRNA gene sequencing

Sample library preparation for Next Generation Sequencing was performed using the QIAseq 16S/ITS Region Panels kit (QIAGEN), targeting the V3V4 hypervariable regions of the bacterial 16S rRNA gene and the fungal Internal Transcribed Spacer (ITS) region.

Libraries were checked through TapeStation 4200 (Agilent Technologies) and quantified by using MicroPlate Reader GloMax (Promega); libraries were then pooled at equimolar concentrations and sequenced on a MiSeq Illumina sequencer; at least 50.000 paired end reads with a length of 275 base pairs (bp) were produced per sample. Quality filtering of sequencing reads was executed with Trimmomatic v0.39, using the following parameter: AVGQUAL:30. Sequences of amplification primers and reads with more than 3 unknown (N) nucleotides were removed using Cutadapt v1.18. High quality and cleaned sequences were analyzed using the Qiime2 platform (v2019.7). Amplicon Sequence Variants (ASVs) were denoised with the Qiime dada2 denoise-paired command setting the following parameters: –p-trunc-len-f 242 –p-trunc-len-r 242. Q2-feature-classifier, trained on the SILVA138 99% OTUs, specifically on the V3V4 region, was used to perform taxonomic classification. All the ASVs classified at least at phylum level were retained for the subsequent analysis. Diversity measures (α- and β-diversity indices) were calculated using the Qiime diversity core-metrics-phylogenetic function with a sampling depth of 3687 sequences. Alpha diversity was evaluated by Shannon and Faith (phylogenic diversity) index and represented by box-and-whisker plot. Community dissimilarities (β-diversity) were evaluated by Bray-Curtis distance and represented by a PCoA plot. Differences of alpha diversity indices across experimental groups were evaluated with Kruskall-Wallis test, correcting for multiple comparisons using statistical hypothesis testing (Dunn's).

QUANTIFICATION AND STATISTICAL ANALYSIS

The sample size for animal studies was guided by previous murine studies in our laboratory. Results are represented using box plots showing the interquartile range, with a single data point per animal; the horizontal lines show the median values, and the whiskers indicate the minimum-to-maximum range. Unless differently specified, samples were never pooled. Statistically significant differences between two groups were evaluated with Mann-Whitney two-sided unpaired U-test, for non-normally distributed data (visually checked), or two-sided unpaired t-test for normally distributed data. When three or more groups were evaluated, Kruskal-Wallis test (non-Gaussian distribution) followed by Dunn's post-test correction, One-Way ANOVA test (Gaussian distribution) followed by Tukey's post-test correction or ANOVA/two-way mixed-effects models followed by Sidak's post-test correction were applied. In case of longitudinal experiments, repeated-measures ANOVA was applied only when the single animal was present in every time point analyzed. Conversely, if different time points included different animals in terminal experiments, conventional ANOVA and mixed-effects models were applied. Following pooled experiments, outliers were detected with the Grubbs' test and excluded from the analysis. A probability value of * $P<0.05$ was considered as significant. All of the statistical details of experiments can be found within figure legends. The investigators were not blinded to the allocation during experiments and outcome assessments. Data analysis and graphing was performed using GraphPad software vers. 9.0.1, LLC.

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Supplemental information

Prenatal antibiotics reduce breast milk IgA and induce dysbiosis in mouse offspring, increasing neonatal susceptibility to bacterial sepsis

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Supplementary Material

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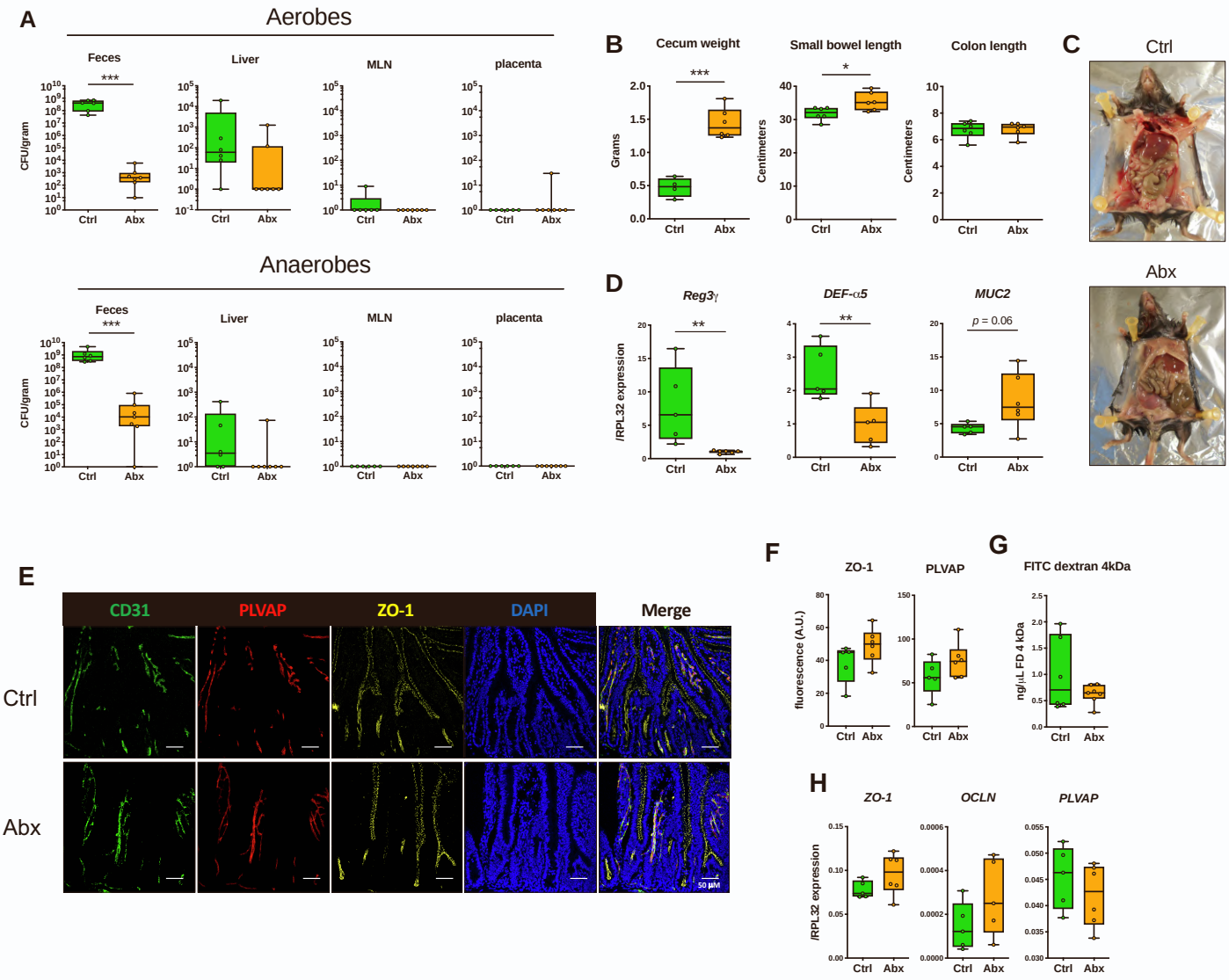
Table S1. Quantification of antibiotics in breast milk of treated dams. Related to Figure 6.

Sample name	Maternal treatment	Volume of Milk (µL)	Erythromycin (ng/ml)	Ampicillin (ng/ml)	Neomycin (ng/ml)
CP8_ABX	**Abx**	20	42.40	< LLOQ (1ng/ml)	< LLOQ (250 ng/ml)
CP8_ABX/CTRL	**Abx**	20	< LLOQ (1ng/ml)	< LLOQ (1ng/ml)	< LLOQ (250 ng/ml)
CP9_ABX	**Abx**	10	< LLOQ (1ng/ml)	< LLOQ (1ng/ml)	< LLOQ (250 ng/ml)
CP10_ABX	**Abx**	10	< LLOQ (1ng/ml)	< LLOQ (1ng/ml)	< LLOQ (250 ng/ml)
POOL_ABX	**Abx**	45	< LLOQ (1ng/ml)	< LLOQ (1ng/ml)	< LLOQ (250 ng/ml)
POOL_ABX/CTRL	**Abx**	5	< LLOQ (1ng/ml)	< LLOQ (1ng/ml)	< LLOQ (250 ng/ml)
CP23_ABX1	**Abx**	20	< LLOQ (1ng/ml)	< LLOQ (1ng/ml)	< LLOQ (250 ng/ml)
CP23_ABX2	**Abx**	20	< LLOQ (1ng/ml)	< LLOQ (1ng/ml)	< LLOQ (250 ng/ml)
CP23_ABX/CTRL	**Abx**	20	< LLOQ (1ng/ml)	< LLOQ (1ng/ml)	< LLOQ (250 ng/ml)

LLOQ: lower limit of quantification as defined by ICH guideline M10 on bioanalytical method validation and study sample analysis - 21 January 2023 (EMA/CHMP/ICH/172948/2019)

Figure S1. Effect of prenatal Abx on maternal small intestine. Related to Figure 1.



A. Amount of culturable aerobic and anaerobic CFUs in maternal organs after 5 days of treatment with antibiotic (Abx) or vehicle (Ctrl). N= 5-8 mice per group, representative of at least 2 independent experiments. P-values calculated by t-test. *<0.05, ***< 0.001.

B. Anatomical parameters in mothers treated or non-treated with antibiotics during late pregnancy. N= 5-8 mice per group, representative of at least 2 independent experiments. P-values calculated by t-test. *<0.05, ***< 0.001.

C. Visual appearance of small and large bowel without or with late-pregnancy antibiotic treatment.

D. Gene expression (relative to housekeeping gene RLP32) of antimicrobial protein/peptides (regenerating islet-derived protein 3 gamma (Reg3γ) and defensin alpha-5 (Defα–5)) and mucus protein (MUC2) by ileal wall in untreated or antibiotic-treated mothers. MLN: mesenteric lymph nodes. N= 5-8 mice per group, representative of at least 2 independent experiments. P-values calculated by t-test. *<0.05, ***< 0.001

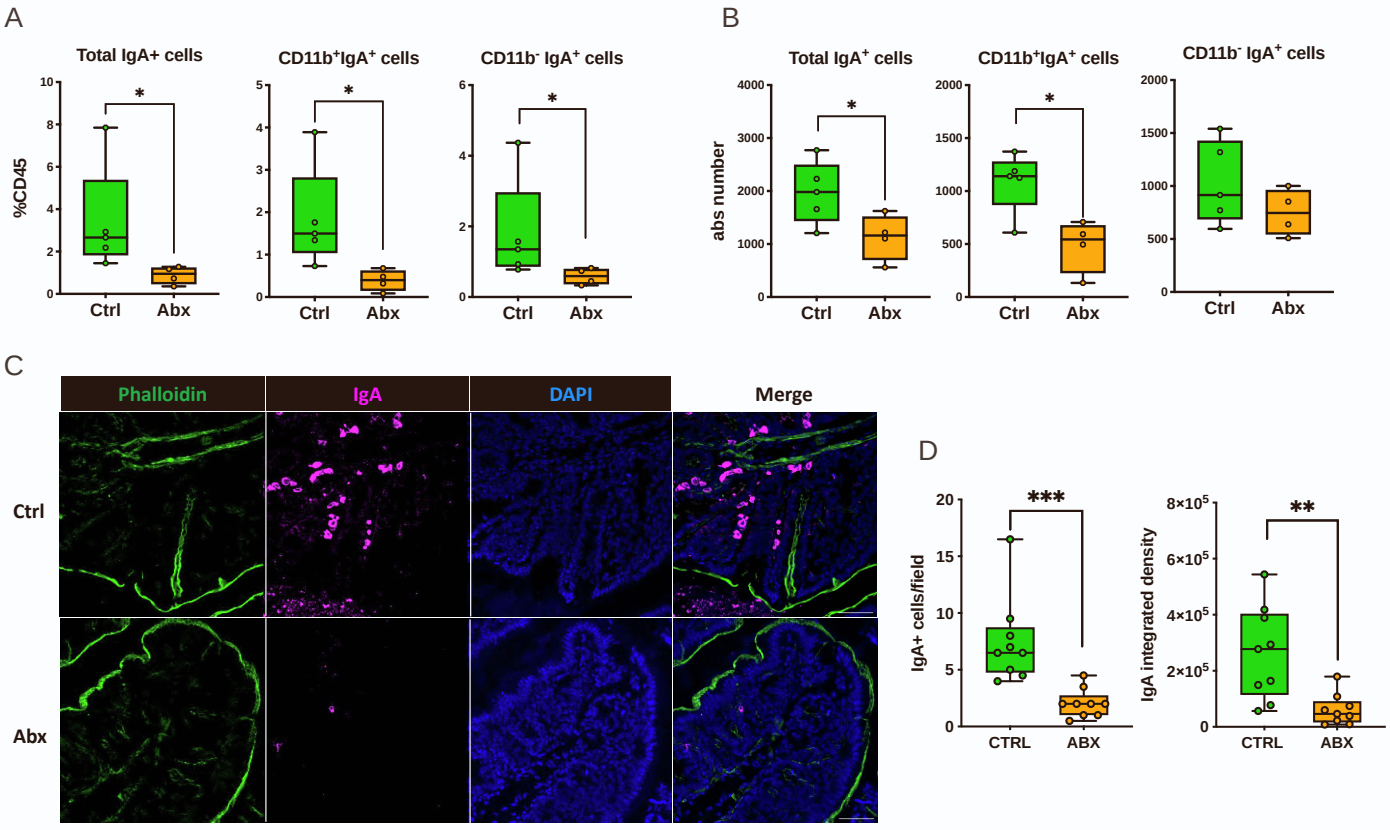
E. Confocal microscopy images of plasmalemma vesicle-associated protein (PLVAP) and zonula occludens.-1 (ZO-1) proteins in maternal ileum at E18.5, with or without Abx treatment.

F. Quantification of ZO-1 and PLVAP fluorescence in (A). N= 4-5 mice per group, representative of at least 2 independent experiments. P-values calculated by t-test

G. Serum concentration of 4 kDa FITC dextran 4 hours after gavage in mothers at E18.5. N= 4-5 mice per group, representative of at least 2 independent experiments. P-values calculated by t-test

H. Gene expression (relative to housekeeping *RPL32*) of tight junction proteins (*OCLN, ZO-1*) and PLVAP in maternal ileum. N= 4-5 mice per group, representative of at least 2 independent experiments. P-values calculated by t-test

Figure S2. Effect of prenatal Abx on neonatal colon. Related to Figure 1.



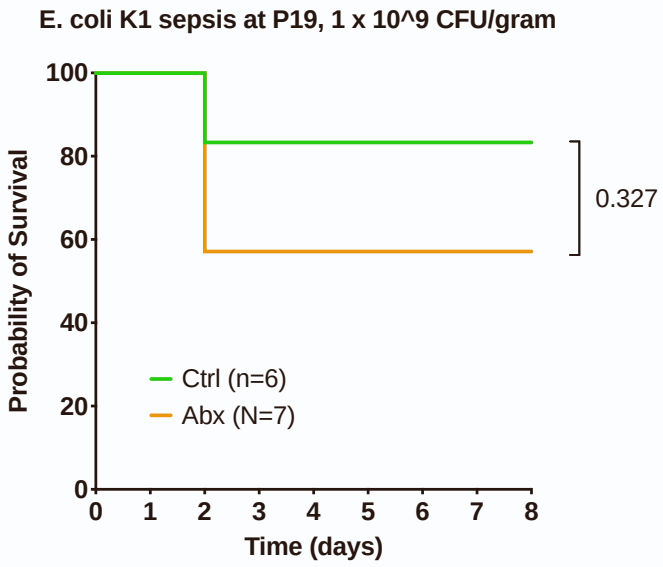
(A-B) Amount of total IgA+ plasma cells and of CD11b+/- subpopulations of plasma cells at P28 in the two experimental groups, expressed as % of CD45+ cells (A) or absolute number per mouse (B) N= 4-5 mice per group, representative of at least 2 independent experiments. P- values calculated by Mann-Whitney U test. *<0.05.

(C) Confocal microscopy images of young adult colon at P28, in the two experimental groups. Representative of 4-5 mice/group.

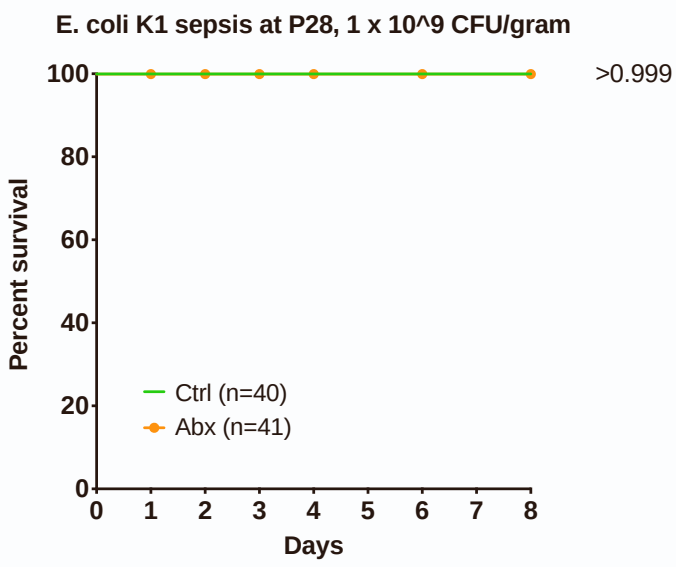
(D-E) Quantification of IgA fluorescence in (C), expressed as number of IgA+ cells/field of view or integrated density. P-value calculated by Mann-Whitney U test, **< 0.01, ***< 0.001.

Figure S3: survival curves of young mice born to Abx-untreated or treated mothers at different ages. Related to Figure 1.

A



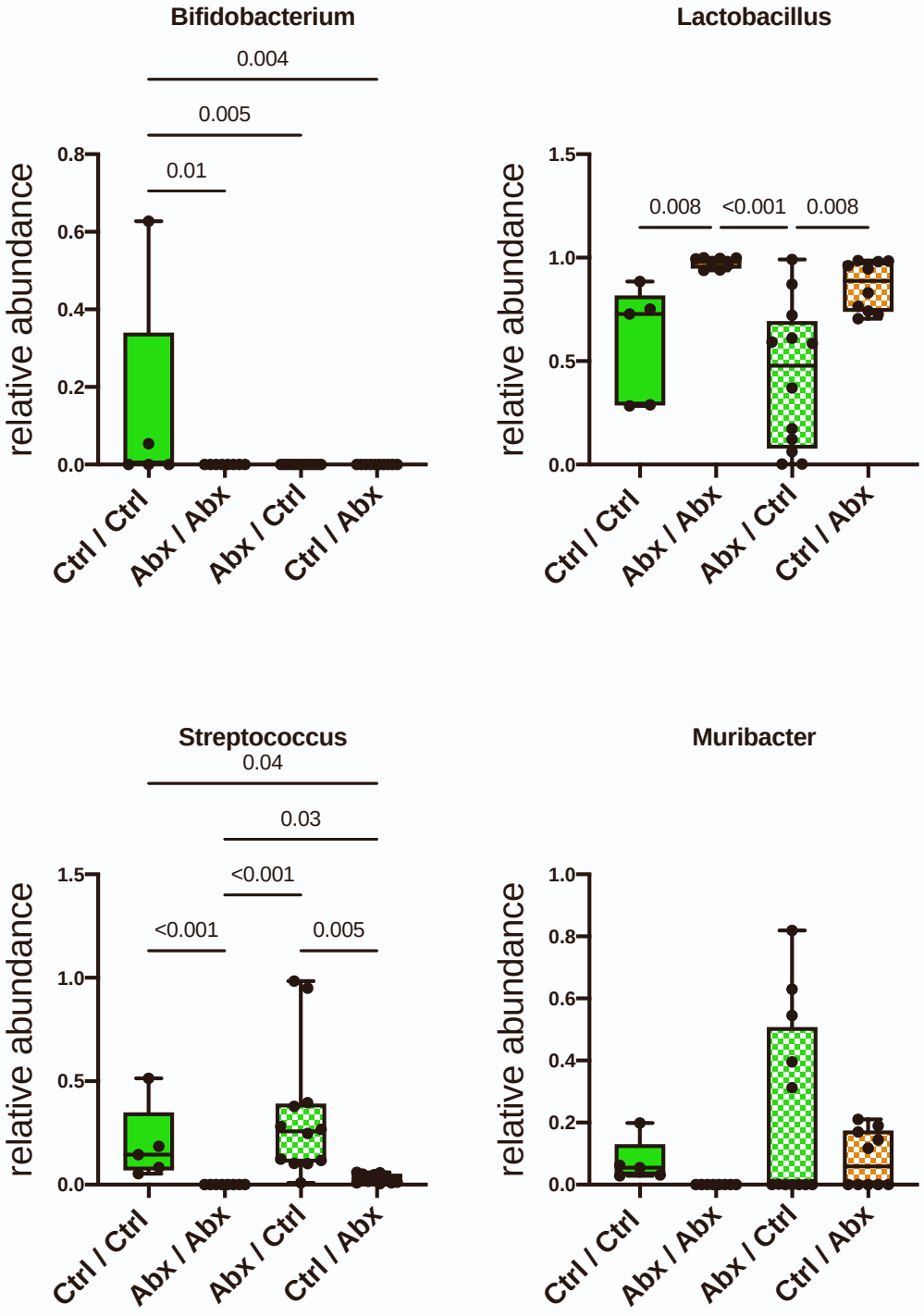
B



(A) Survival curves at P19 of mice born to Abx-treated or -untreated mothers and challenged with E. coli K1. Representative of 3 independent experiments.

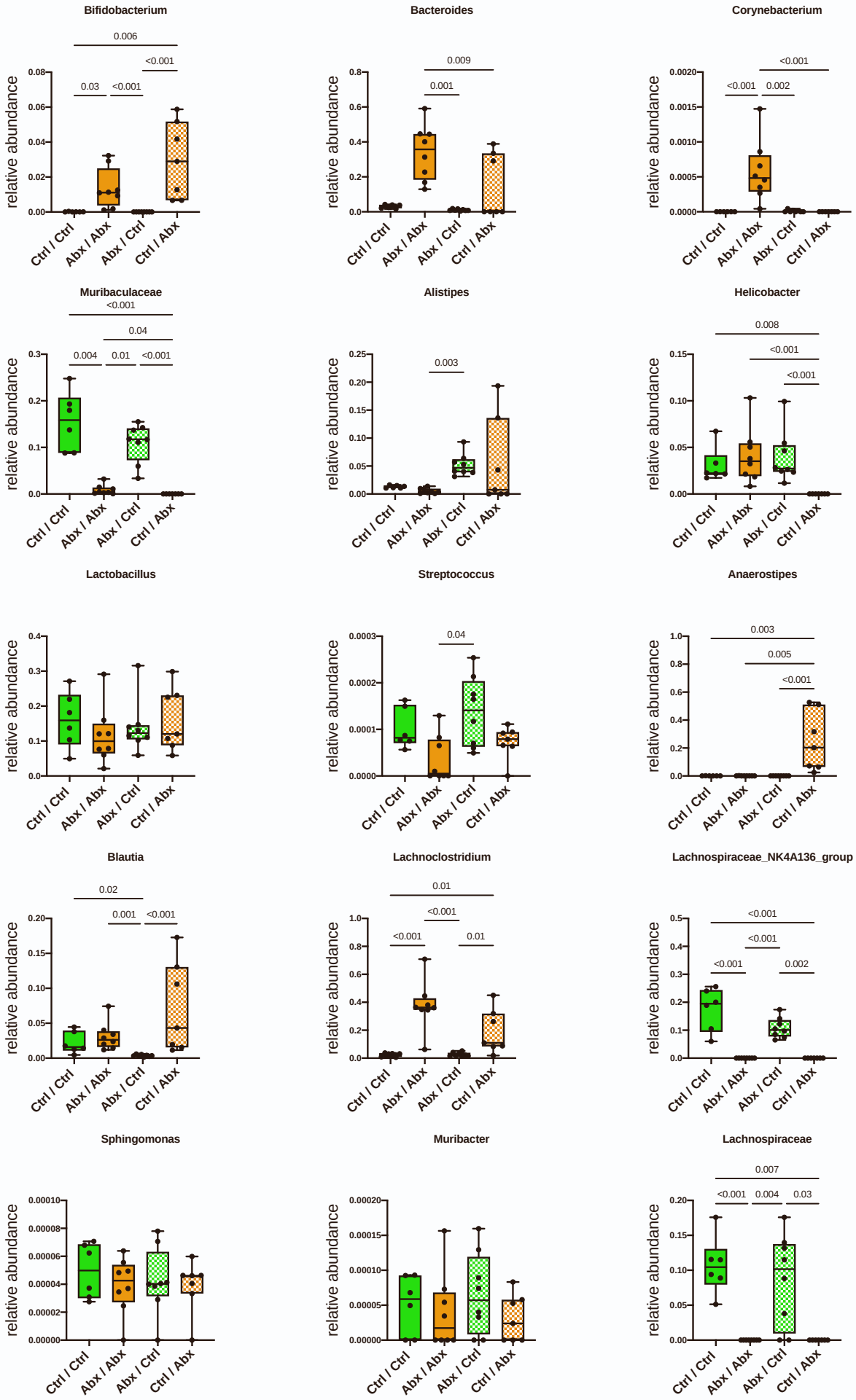
(B) Survival curves at P28 of mice born to Abx-treated or -untreated mothers and challenged with E. coli K1. Pooled from 3 independent experiments.

Figure S4. Relative abundance of bacterial genera found in fecal pellets from pups of the 4 experimental groups at P7. Related to Figure 6.



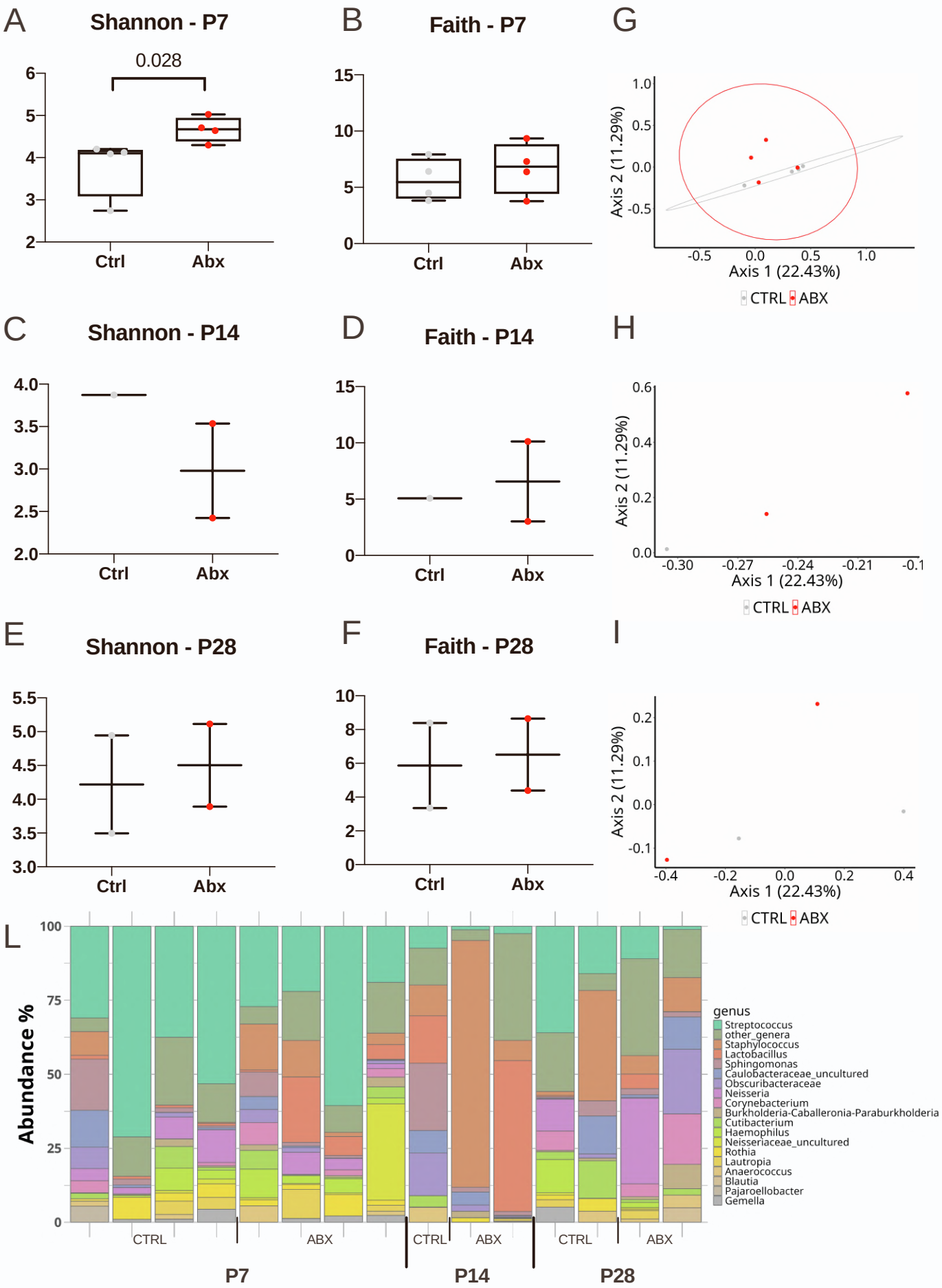
P values calculated by Kruskall Wallis test with two-stage step-up method (Benjamini, Krieger and Yekutieli) of correction to control the False Discovery Rate.

Figure S5. Relative abundance of bacterial genera found in fecal pellets from pups of the 4 experimental groups at P28. Related to Figure 6.



P-values calculated by Kruskall Wallis test with two-stage step-up method (Benjamini, Krieger and Yekutieli) of correction to control the False Discovery Rate.

Figure S6. Influence of prenatal antibiotics on mammary gland microbiota. Related to Figure 6.



(A) Alpha-diversity (Shannon index) of mammary gland microbiota at P7 in the 2 experimental groups of pups.

(B) Alpha-diversity (Faith index) of mammary gland microbiota at P7 in the 2 experimental groups of pups.

(C) Alpha-diversity (Shannon index) of mammary gland microbiota at P14 in the 2 experimental groups of pups.

(D) Alpha-diversity (Faith index) of mammary gland microbiota at P14 in the 2 experimental groups of pups.

(E) Alpha-diversity (Shannon index) of mammary gland microbiota at P28 in the 2 experimental groups of pups.

(F) Alpha-diversity (Faith index) of mammary gland microbiota at P28 in the 2 experimental groups of pups.

(G-I) Beta-diversity (Bray Curtis principal component analysis) of mammary gland microbiota at P7-14-18 in the 3 experimental groups).

(L) Barplots depicting the bacterial general of mammary gland in the 2 experimental group (indicated as mother/pups) at P7-14-28.

For A-L, N= 1-4 mice per group. P- values calculated by Mann-Whitney U test. *<0.05