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Prenatal antibiotic therapy predisposes to neonatal late-onset sepsis induced by E.coli: a role for breast milk IgA?

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List of Abbreviations

- **Abx**: antibiotics
- **APC**: antigen-presenting cell
- **CD**: cluster of differentiation
- **DCs**: dendritic cells
- **ELISA**: enzyme-linked immunosorbent assay
- **FACS**: flow-cytometry activated cell sorting
- **FBS**: fetal bovine serum
- **Foxp3**: forkhead box P3
- **GI:** gastrointestinal
- **iFOS:** immunoFOS®
- **IgA**: immunoglobulin A
- **IL**: interleukin
- **ILC**: Innate Lymphoid Cells
- **LI**: large intestine
- **LP**: lamina propria
- **MDX**: maltodextrins
- **MNPs**: mononuclear phagocytes
- **P**: postnatal day
- **PBS**: phosphate buffer saline
- **PC**: plasma cell
- **RORγt**: RAR-related orphan receptor gamma, isoform t
- **ROS**: reactive oxygen species
- **SCFAs**: short chain fatty acids
- **SI**: small intestine
- **sIgA**: secretory IgA
- **T-reg**: T-regulatory lymphocyte
- **Th**: T-helper lymphocyte
- **TMB**: 3,3',5,5'-Tetramethylbenzidine

Abstract

Antibiotics are administered to 20-30% of pregnant women, to treat maternal infections or to prevent fetal and neonatal bacterial colonization in selected cases of high infection risk, such as premature rupture of membranes (PROM). Despite the administration of antibiotics to neonates, i.e. in the first days of life, is known to increase the risk for subsequent late-onset sepsis (LOS), necrotizing enterocolitis (NEC), and possibly of long term adverse outcomes such as obesity and allergic manifestations, the effect of prenatal antibiotics on neonatal immune development and function is poorly understood. Here, using a murine model of prenatally-restricted antibiotic therapy (Abx), we show that newborn mice born to mothers previously treated with Abx are more susceptible to LOS caused by *Escherichia coli*, in correlation with an increased bacterial translocation to distant organs (mesenteric lymph nodes, spleen, liver). Increased neonatal mortality after maternal Abx did not correlate with major modifications in blood or spleen populations of immune cells (neutrophils, activated monocytes, $CD4^+$ and $CD8^+$ T cells), nor with changes in gut epithelial structure and integrity, but correlated with a significantly lower amount of fecal IgA, stomach IgA, IgA-coating of intestinal bacteria, and IgA production by plasma cells of the maternal mammary gland. Cross fostering experiments reinforced the evidence that low breast milk IgA after maternal Abx was a key factor to increase the mortality rate of pups. Moreover, the adverse effect of prenatal Abx persisted after the interruption of breastfeeding, in weaned young adults. At 28 days of life, prenatal Abx caused a reduction in IgA^+ plasma cells, both $CD11b^+$ and $CD11b^-$, in the lamina propria (LP) of the ileum, a reduction in faecal sIgA, and decreased the amount of colonic T-regs and of Th17-like lymphocytes

($CD4^+$ $ROR\gamma t^+$ antigen-specific T cells). Maternal supplementation with postbiotics during the last 5 days of pregnancy was not sufficient to revert the antibiotic-induced phenotype, at least in our experimental setting. Our data highlight the importance of breast milk IgA for the prevention of neonatal LOS, and the influence of prenatal Abx. If confirmed on human samples, these results may lay the foundation for screening strategies of IgA in breast milk, especially in selected high-risk situations such as prenatal Abx administration, and/or extremely preterm neonates receiving maternal (or donor) breast milk.

Chapter 1: Introduction

Early life is a period of rapid and massive transformations as no other in the life of mammalians, and the immune system is no exception to this. In particular, the immune system has to deal with the impressive challenge of managing for the first time thousands of environmental microorganisms, both pathogenic and harmless, and to correctly build up in a short time the community of commensal bacteria, fungi and viruses known as microbiome, mycobiome, and virome, respectively **(Al Nabhani and Eberl, 2020; Kalbermatter et al., 2021)**. This huge task requires a unique and precisely regulated set-up of several immune functions, both systemically and at the mucosal sites, where most of microbiome-host interactions take place **(Zhang et al., 2017)**. Interestingly, over the last few years it has become clear that the interaction between the neonatal immune system and the assembling microbiome is not unidirectional, but reciprocal **(Sanidad and Zeng, 2020)**. Indeed, the components of early life microbiome can affect the correct development and function of several immune populations, both systemic, as circulating neutrophils, or at the mucosal level, such as colonic T-regulatory $CD4^+$ lymphocytes (Tregs) or innate lymphoid cells (ILCs) of the small intestine **(Ansaldo et al., 2021)**.

Several environmental factors can affect this microbiome-immune system interaction (**Figure 1**). Among them, two have the potential of dramatically shifting the developmental trajectories of neonatal immune system, both before and early after birth: nutrition, both maternal and neonatal, and antibiotic therapies administered to the mother or the neonate. The effects of these two factors on neonatal immune function, either direct or through modifications of the microbiome, are only partially understood.

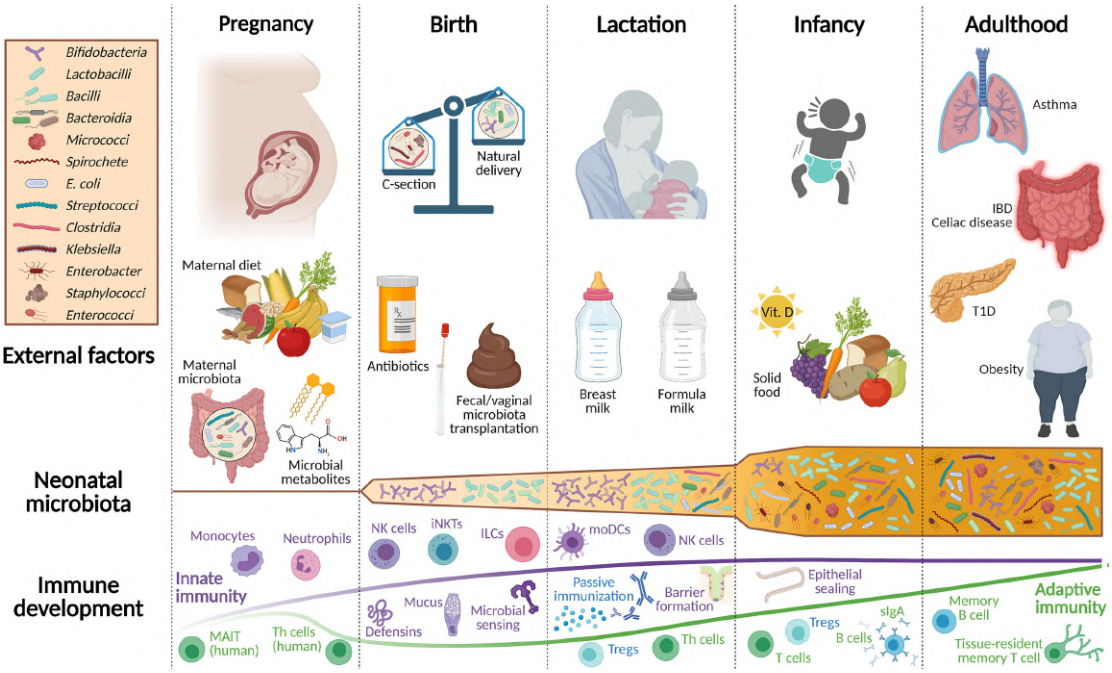

Figure 1. Overview of environmental factors shaping the development of the newborn microbiota and mucosal immune system. iNKTs: invariant NK-T cells. NK: natural killer cells. ILCs: innate lymphoid cells. MoDCs: monocyte-derived dendritic cells. MAIT: mucosa-associated invariant T-cells. Reproduced from Kalbermatter C, Front Immunol 2021 **(Kalbermatter et al., 2021)**

1.1 Characteristics and development of the neonatal immune system

1.1.1 Neonatal innate systemic immunity

Both innate and adaptive components of the immune system present unique characteristics during the neonatal period. At birth, the number of neutrophils ranges can vary widely, from 1.5–28 × 10^9 cells/L blood, compared to steady state levels of 4.4 × 10^9/L in adults, but the storage pools and the number of neutrophil progenitor cells are lower in neonates than in adults, leading to diminished neutrophil responses to infection **(Melvan et al., 2010)**. Besides the quantitative deficiencies, neonatal neutrophils have lower surface expression levels of the major lipopolysaccharide (LPS) receptor, the toll-like receptor (TRL) 4, defective downstream signaling through MyD88 and p38

pathways, lower levels of cell surface L-selectin and CD11b, which mediate the rolling and adhesion of cells to the vascular endothelium, and are defective in making Neutrophil Extracellular Traps (NETs) used to kill extracellular bacteria **(Anderson DC, 1990; Araujo et al., 2016; Filias et al., 2011; Raymond et al., 2017; Yost et al., 2016)**.

Natural killer (NK) cells play a key role in controlling infections caused by viruses and some intracellular bacteria. Although their number in umbilical cord blood is comparable or even greater than that of adults, a significantly higher percentage of cells with immature phenotype ($CD56^{low}$ $CD57^{low}$) is observed, and the maturation delay of this cell line appears to be mediated in part by elevated levels of transforming growth factor (TGF) -β in neonatal plasma **(Dalle et al., 2005)**. From a functional point of view, neonatal NK cells have a reduced number of cytoplasmic granules, reduced degranulation capacity with consequent reduced cytolytic activity (40-50% compared to adults) and a lower production of IFN-γ **(Ivarsson et al., 2013)**.

Dendritic cells (DCs), the prototypical antigen-presenting cells (APCs), process and present antigens to T lymphocytes: several scientific evidences have shown that both antigen recognition and presentation to T lymphocytes, as well as their subsequent activation, are altered in neonatal DCs compared to adults **(Dowling and Levy, 2014)**. Conventional neonatal DCs (cDCs, $MHCII^{+}$, $CD11c^{+}$, $CD14^{-/low}$, $CD123^{-}$), even more those of premature newborns, express lower quantities of both MHCII and co-stimulatory molecules such as CD40, CD80 and CD86 on their cell surface after stimulation **(Nguyen et al., 2010)**. It is also known that newborn DCs produce high quantities of IL-6 and IL-10, but considerably reduced quantities of IL-12p70 compared to adults, largely due to a transcription defect of the 12p35 subunit **(Goriely et al., 2004; Kollmann et al., 2017)**. This different cytokine pattern in the newborn causes an imbalance in the polarization of T-helper (Th) lymphocytes in favor of Th2,

Th17 and T-regulatory functional lymphocytes (Tregs), at the expense of Th1 polarization, with subsequent lower production of IFNγ by the CD4+ T cells themselves and, in turn, less effective antiviral responses by $CD8^+$ T cells, diminished antigen-specific antibody production by B lymphocytes, less activation of NK cells and macrophages **(Debock and Flamand, 2014; Lee et al., 2008)**.

1.1.2 Neonatal adaptive systemic immunity

Most neonatal T-cells have an immature ($CD38^{+)}$ and naïve ($CD45RA^+$ $CD45RO^-$) phenotype, with characteristics attributable to the so called "recent thymic emigrants" and high levels of DNA residues derived from rearrangements of the T-cell α-chains (the so-called "TRECs") **(Marchant and Goldman, 2005)**. The distribution of T lymphocyte subpopulations is different in the newborn compared to the adult, and the $CD4^+/CD8^+$ ratio is unbalanced in favor of $CD4^+$ **(Adkins et al., 2004)**. Furthermore, $CD4^+$ $Foxp3^+$ Treg cells represent about 12% of total cord blood T cells, a much higher proportion compared to adults, reflecting a clearly skewed setting of fetal adaptive immune system. As mentioned before, following stimulation by APCs, neonatal $CD4^+$ T cells show a predominant production of Th2-Th17-Treg pool of cytokines and chemokines **(Debock and Flamand, 2014)**, with upregulation of the corresponding transcription factors (GATA3, RORγt and Foxp3, respectively). In addition to the different stimulation by APCs, neonatal $CD4^+$ T lymphocytes have an intrinsic deficiency in INFγ production, due to the hyper methylation of the IFN gene promoter compared to adults **(White et al., 2002)**. Similarly, but in the opposite functional direction, the hypo methylation of CNS-1 promoter favors the transcription of Th2-associated genes **(Zaghouani et al., 2009)**. For what concerns cytotoxic $CD8^+$ T cells, they also show function differences in neonates compared to adults. In particular,

it has been shown by different researchers that neonatal $CD8^+$ T cells, rather than being "defective", present a clear transcriptional polarization towards innate immune functions, with lower transcription of antigen-specific T-cell receptor (TCR) activation-related genes and increased transcription of genes connected to innate functions, such as that of TLR5 **(Galindo-Albarran et al., 2016; Siefker and Adkins, 2016)**.

Finally, antibody production by neonatal B-cells in early life is almost absent. This defect is multifactorial. First, it is a natural consequence of the limited antigenic exposure in utero, of the "inhibitory" presence of maternal antibodies **(Koch et al., 2016)**, and on the weak stimulation by T-follicular helper cells in the germinal centers of lymphoid organs **(Pietrasanta et al., 2021)**. Secondly, this defect is also attributable to some intrinsic characteristics of neonatal B lymphocytes: in particular, a predominantly immature membrane phenotype (IgM^+, IgD^-), and a hypo valid signaling of the B cell receptor (BCR) **(Tian et al., 2006)**.

1.1.3 Neonatal mucosal immunity: the intestine

The mucosal sites of the body, in particular the gastrointestinal (GI) tract and the lung, are non-sterile sites where a continuous and intense interaction between the microbiome and several components of the immune system takes place **(Ansaldo et al., 2021)**. Considered that neonates come to life with an incomplete maturation of mucosal immune function, and from an environment poor of antigenic stimulation (but possibly not completely devoid **(Mishra et al., 2021; Perez-Munoz et al., 2017)**) like the womb, this interaction in early postnatal life resembles a roaring fire.

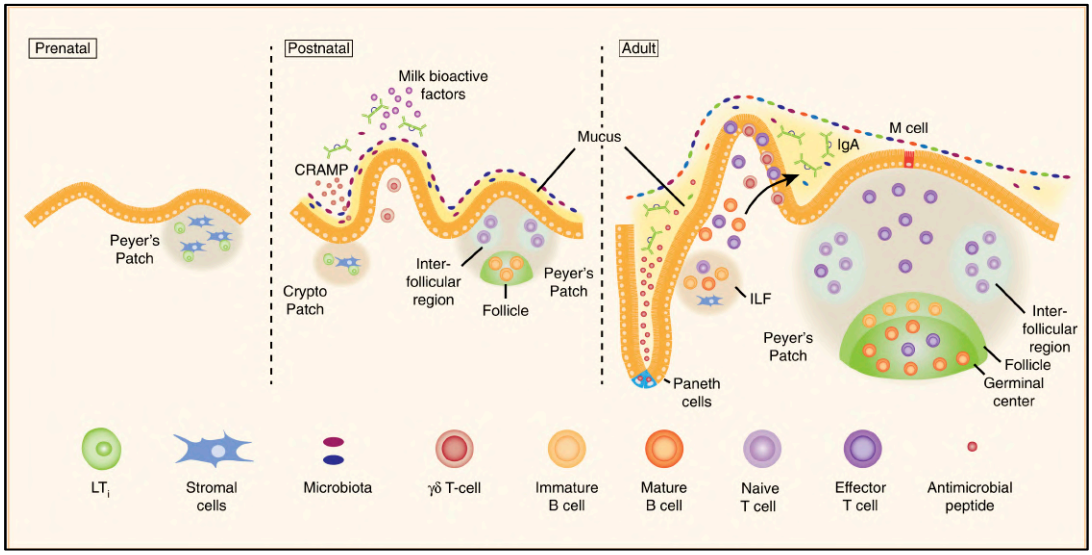

Figure 2. Development of the intestinal mucosal immune system. LTi: lymphoid tissue inducers. CRAMP: cathelicidin-related antimicrobial peptide. Tregs: regulatory T cells. ILFs: isolated lymphoid follicles. SigA: secretory immunoglobulin A. Reproduced from Torow N, Mucosal Immunol 2017 **(Torow et al., 2017)**

Several components of the innate immune system reside in the gut, and take part to the developmental processes of early life (**Figure 2**). Paneth cells, that produce antimicrobial peptides as α-defensins, lysozyme, secretory phospholipase A and serum amyloid A, are not completely developed at birth, and their maturation requires the interaction with components of the microbiota and with food antigens **(Bry et al., 1994)**. Nonetheless, the expression of the cathelicidin-related antimicrobial peptide (CRAMP) has been reported in the neonatal intestinal epithelium, as well as that of other antimicrobial proteins, as the Regenerating islet-derived protein 3 gamma (Reg3γ), or mucus proteins (e.g. muc2, muc3, muc5), produced by globet cells. Together, these represent a fundamental defensive component of the neonatal intestinal barrier **(Hornef et al., 2004; Menard et al., 2008)**. Tissue resident intestinal macrophages (MΦs, CX3CR1^{hi} CD64^{+} MHCII^{+} cells), that patrol the intestinal lumen taking up food and bacterial antigens, are already present before birth **(Bain et al., 2014; van de Laar et al., 2016)** in the GI mucosa, and cooperate with intestinal dendritic cells (DCs, CD11b^{+} CD11c^{+} CD103^{+}) for the final transfer of antigens to mesenteric lymph nodes (MLNs). Intestinal DCs are also present in the postnatal intestine **(Torow et al., 2015)**. Before birth,

Peyer's patches precursors of the small intestine (SI) develop within the sterile, or quasi-sterile, environment of the womb via a crosstalk between lymphoid tissue inducer cells (LTi) and the stroma cells **(Honda et al., 2001)**. LTi cells are a subsets of innate lymphoid cells (ILCs), the type 3 (ILC3), that express the RAR-related orphan receptor γt (RORγt) and are capable of supporting the maturation of mucosal lymphoid sites (like Peyer's patches) thanks to the expression of the chemokine receptors CXCR5 and CCR7 **(Eberl et al., 2004)**. Several factors have been shown to influence the functionality of ILC3, and the consequent maturation of intestinal lymphoid sites, both before and after birth, such as maternal nutrition during pregnancy, breastfeeding, and the occurrence of infections **(Gomez de Agüero et al., 2016; Lim et al., 2021)**.

The SI mucosa is also populated by adaptive immune cells, in particular intraepithelial lymphocytes (IEL). Fetal IEL are exclusively γδ T-cells, progressively replaced by αβ T-cells starting from birth **(Haas et al., 2012; Ramond et al., 2014)**. IEL in early life are mostly confined within the Peyer's patches, without significant colonization of the effector sites like the mucosa, and include thymus derived regulatory $CD4^+$ T cells (tTregs), T-helper $CD4^+$ T cells, and B lymphocytes. In particular, Tregs have the fundamental role of enforcing the tolerance towards bacterial commensal antigens, self-antigens and food antigens already shortly after birth **(Hadis et al., 2011)**. As it happens for circulating lymphocytes, the proportion of mucosal naive T cells in neonatal patients is significantly higher than in adults. Despite B cells populate the intestine soon after birth, the occurrence of fully functional plasma cells and the complete repertoire formation of secretory immunoglobulin A (SIgA) production are only noted after weaning **(Harris et al., 2006; Lindner et al., 2012)**.

1.2 How nutrition and the microbiome can affect the development of immune system

1.2.1 Prenatal nutrition

What a pregnant mother eats, both quantitatively and qualitatively, can profoundly affect the development of her fetus **(Obanewa and Newell, 2017)**. Several robust studies have shown that maternal protein and energy malnutrition (PEM) is a major cause of intrauterine growth restriction (IUGR), can cause preterm birth, and, interestingly, can severely compromise the maturation of the fetal immune system, with consequences in childhood independently from an adequate postnatal nutrition **(Grindulis et al., 1984; Xiao et al., 2015)**. Human infants with IUGR are more likely to have evidence of significant impairment of both cell-mediated and humoral immunity than infants born appropriate for gestational age (AGA), and these effects can last long after birth **(Chandra, 1979)**. Furthermore, even without the macroscopic evidence of IUGR, nutritional deficiencies during pregnancy can lead to a delayed maturation and suboptimal function of the neonatal immune system. For example, several micronutrients (minerals or vitamins) are known to be important to maintain a physiological development of the fetal and neonatal immune system. Zinc, which is obtained from the consumption of meat and legumes, is important to guarantee the structure of DNA and protein, and to support nutrient metabolism **(Murakami and Hirano, 2008)**. Lack of zinc can impair resistance to bacterial infection, reduce both thymic and spleen size and function, T-lymphocyte development, and T-cell-dependent B-cell functions **(Beach et al., 1982)**. Clinically, randomized controlled trials in resource-limited countries have shown that maternal zinc supplementation has a beneficial effect on neonatal immune status, and in particular on early

neonatal morbidity due to infections **(Fischer Walker and Black, 2004)**. Another example is the fat-soluble vitamin A assumed with whole milk, liver, eggs, dark colored fruits and vegetables. Vitamin A is essential in maintaining the integrity of mucosal surfaces, which are important for the interaction with microbiome and for oral vaccine immune responses (e.g rotavirus vaccine), and for the formation of secondary lymphoid organs by inducing the differentiation of LTi cells **(Bhaskaram and Rao, 1997; Goverse et al., 2016)**. Vitamin D, whose fetal stores are highly dependent on maternal nutritional levels **(Kovacs, 2008)**, is known to promote Th2 and Treg signaling, and to increase macrophages and monocytes anti-mycobacterial effects. Vitamin D is a potent enhancer of the suppressive capacity of Treg cells, and can control Th1 immunity, a key mechanism involved in maternal–fetal rejection **(Bikle, 2008)**. Interestingly, a strong body of evidence has confirmed that also maternal over nutrition and obesity take their toll on the fetal immune system, being associated with increased rates of preterm birth, and with several inflammatory and metabolic diseases in the offspring, including obesity itself and asthma **(Godfrey et al., 2017) (Shankar et al., 2008)**.

1.2.2 Prenatal microbiome

If macro-and micro-nutrients in the maternal diet can affect the development and function of early life immune system, this is also true for other "occupants" of the maternal gastrointestinal tract: the intestinal microbiota.

The effect of maternal microbiome on fetal and neonatal immune system can be indirect, through the influence of bacteria on nutrients in the GI tract, or direct, due to the passage of bacterial components, metabolites, and possibly also of live microorganisms **(Macpherson et al., 2017)** in the maternal circulation and to the developing fetus.

Different nutrients-mediated effects of maternal microbiome on immune system have been demonstrated. First, intestinal microorganisms can increase the energy harvest from the diet by fermenting food components resistant to mammalian enzymes, such as complex carbohydrates, producing metabolites like short-chain fatty acids (SFCAs) that represent an important energy source for the cells of the intestinal epithelium **(Flint et al., 2012)**. Second, SCFAs provide a significant contribution to different functions of the immune system. Indeed, SCFAs can alter the chemotaxis and phagocytosis of macrophages, induce reactive oxygen species (ROS), change cell proliferation and function, and have anti-inflammatory, anti-tumorigenic, and antimicrobial effects **(Schulthess et al., 2019; Silva et al., 2020)**. Third, components of the intestinal microbiota can synthetize essential vitamins that mammalian organisms

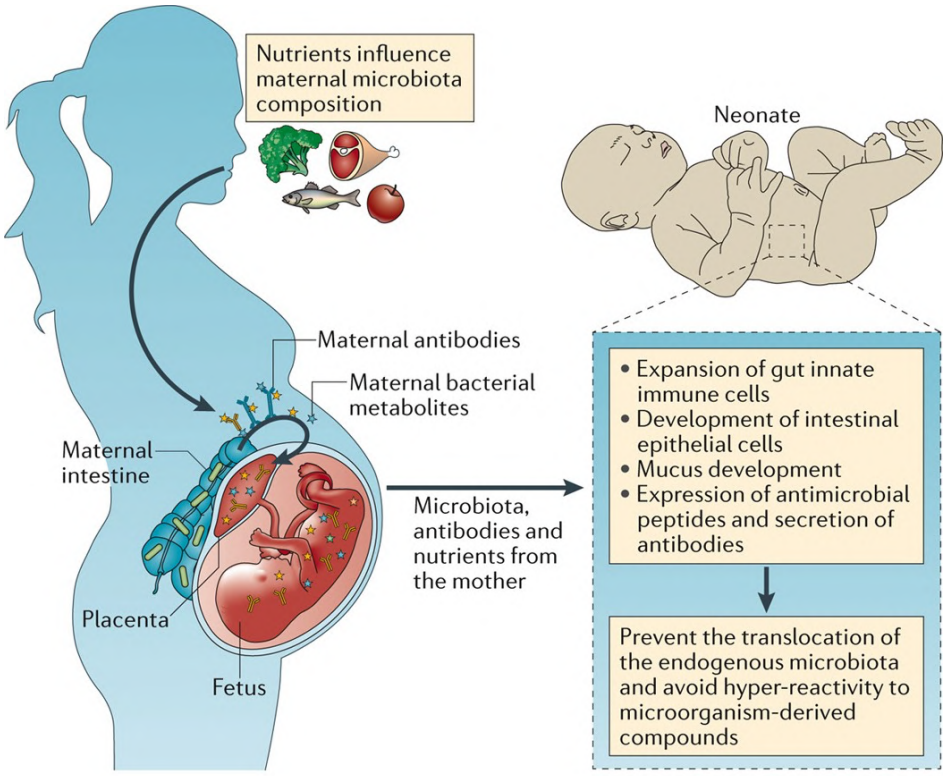

Figure 3. Schematic of the effects of the maternal microbiota and maternal nutrition on immune development in the offspring. Reproduced from Macpherson AJ, Nat Rev Immunol 2017 **(Macpherson et al., 2017)**

cannot produce, or produce only in limited amounts, such as vitamin K, essential for the synthesis of coagulation factors, folate, or vitamin B12 **(LeBlanc et al., 2013)**.

The direct effect of maternal microbiome on the developing fetus, and the subsequent effects on the maturation of the immune function, are among the most fascinating and debated issues in modern developmental immunology (**Figure 3**). Although the biomass of the host and of its microbiota are rather well separated, and this holds even more true for the fetus during the gestation thanks to the placental barrier, the separation is not absolute. Significant amounts of microbial components and few live, whole microorganisms can be found in body sites traditionally considered as "sterile", including the womb **(Ganal-Vonarburg et al., 2020)**. A seminal work by the MacPherson group **(Gomez de Agüero et al., 2016)** has recently shown that transient colonization of germ-free pregnant female mice can shape the immune system of the offspring, increasing the absolute number of both intestinal ILC3 and $F4/80^{+}CD11c^{+}$ mononuclear cells, making pups more resistant to antimicrobial inflammatory responses and bacterial translocation from the intestine. Mechanistically, the effect was mainly caused bacterial metabolites of the maternal intestinal microbiota entering the maternal circulation and being carried through the placental barrier by maternal IgG. Furthermore, the Ginhoux research group has recently demonstrated **(Mishra et al., 2021)** the presence of few but consistent bacteria in several body sites of human fetuses since 14 weeks of pregnancy, demonstrating how these cells are able to induce the activation of memory T-cells *in vitro*.

Finally, several clinical studies in human subjects have shown promising protective effects of nutritional supplementation with probiotics, i.e. live commensal bacteria, of pregnant mothers at high risk to give birth to an allergic/atopic baby **(Dotterud et al., 2010; Rautava et al., 2012; Wang et al.,**

2019). Whether this effect is due to 1) a direct passage of some of these probiotics to the fetus during pregnancy, with a subsequent modulation of the fetal systemic immune response, 2) the passage of bacterial metabolites through the placenta, 3) a predominant effect of modulation of the maternal immune system, or 4) a combination of these mechanism, has to be established yet. Furthermore, there is still a great deal of uncertainty concerning the most appropriate probiotic strain to use, as well as the correct dose, and the timing of administration **(Fiocchi et al., 2015)**.

1.2.3 postnatal nutrition and the role of breast milk

After birth, another important actor comes into play to modulate the maturation of neonatal immune system, both mucosal and systemic, and the progressive acquisition of postnatal microbiome: the breast milk. Breast milk represents the natural and more appropriate nutritional source for neonates during the first months of life, but its role goes far beyond the intake of macronutrients and calories to the growing baby **(Victora et al., 2016)**. Breast milk is a rich source of antimicrobial substances, including immunoglobulins, predominantly sIgA produced by plasma cells (PCs) of the mammary gland, complement proteins, lysozyme, and lactoferrin. These factors play a role in the protection against a wide range of pathogens, and can support the neonatal immune system against the occurrence of early-life infections. As stated, human milk constituents can also directly promote the maturation of the neonatal immune system, through several mechanisms **(Gregory and Walker, 2013)**. First, breast milk is a means to directly transfer immune cells, such as macrophages, neutrophils, and lymphocytes, from the mother to the infant. These cells can directly elicit immune responses, but possibly influence the phenotype of neonatal immune cells, especially B and T cells **(Witkowska-Zimny and Kaminska-El-Hassan, 2017)**. Second, a variety of

immunologically-active substances are also transferred via breast milk. These compounds include hormones (e.g., estrogen and progesterone), growth factors (e.g., epidermal growth factor), and cytokines, such the IL-10 and transforming growth factor - beta (TGF-β) produced by mammary epithelial cells. It has been suggested that mammary gland-derived IL-10 ad TGF-β are involved in the induction of neonatal tolerance towards both dietary and microbial antigens **(Faria and Weiner, 2006)**. At the same time, breast milk is also a source of several proinflammatory cytokines, including tumor necrosis factor (TNF), IL-1β, IL-6, and IL-8, but all in lower amounts as compared with anti-inflammatory and tolerogenic compounds **(Ferrari et al., 2020)**. Third, breast milk is an important (and exclusive) source of indigestible polymers of simple sugars named "human milk oligosaccharides", or HMOs. HMOs are the third most abundant solid component of human milk, after lactose and fat, and are known to have several immuno-modulatory functions **(Bode, 2018)**. For example, HMOs can act as anti-inflammatory molecules, by inhibiting the formation of platelet–neutrophil complexes, they have prebiotic functions, promoting the growth of intestinal *Bifidobacteria*, and they can prevent the adhesion of pathogens to the intestinal epithelium acting as "decoy" receptors, for a variety of pathogenic bacteria **(Donovan and Comstock, 2017)**. Fourth, human breast milk harbors its own microbiota, virome, and mycobiome, which are also transferred to the infant and are involved in immune maturation both directly and indirectly, by shaping the constituents of the intestinal microbiota **(Moossavi et al., 2019)**. Breast milk microbiome is largely derived by the maternal gut microbiome, whose components can translocate through enterocyte tight junctions (that are more permeable during the last stages of pregnancy and during lactation), are taken up by mononuclear cells and travel to the mammary gland through the so called "entero-mammary pathway" (**Figure 4**) **(Moossavi and Azad, 2020)**.

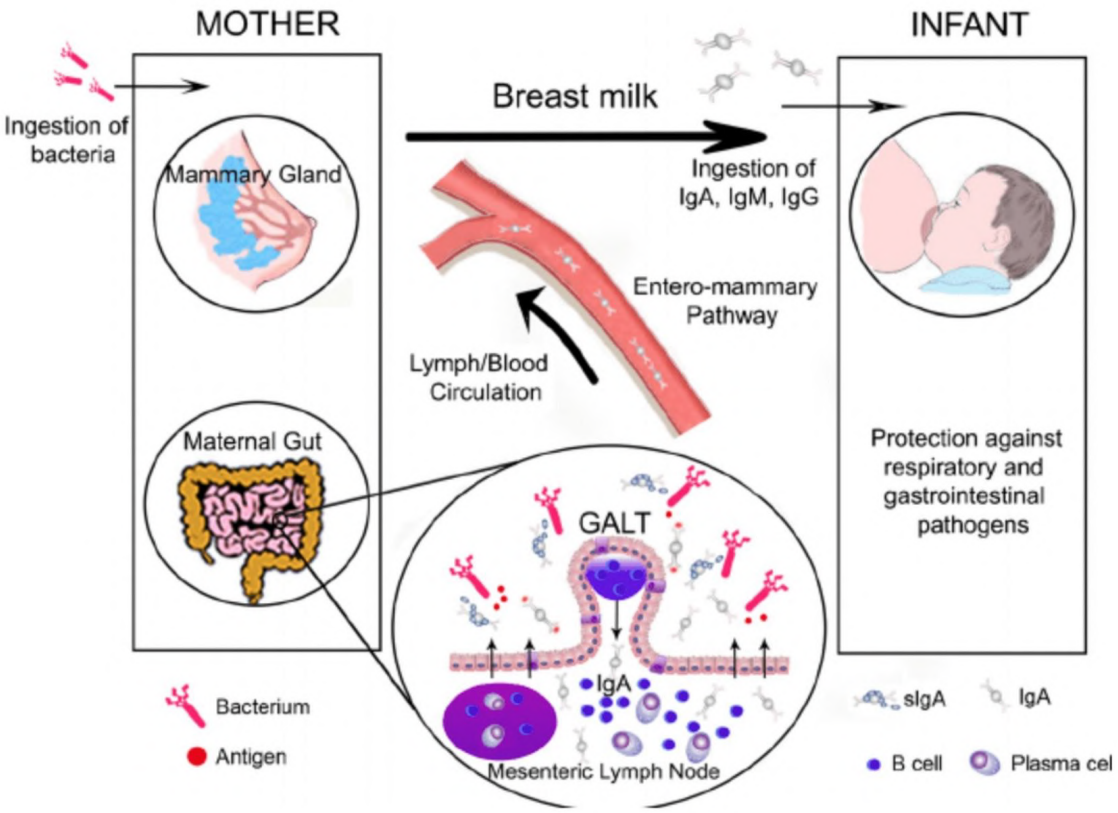

Figure 4. Pictorial representation of the entero-mammary pathway. GALT: gut-associated lymphoid tissue. sIgA: secretory immunoglobulin A.

Despite significant inter-individual variations due to genetic and environmental factors, some bacterial strains are commonly isolated in human milk samples, including *Bifidobacterium, Lactobacillus, Staphylococcus, Streptococcus, Bacteroides, Clostridium, Micrococcus, Enterococcus,* and *Escherichia*. Fifth, as previously mentioned, antibodies are transferred in large quantities from the mother to the breastfed neonate through breast milk **(Atyeo and Alter, 2021)**.

In particular, the most ab undant immunoglobulins in breast milk are secretory IgA (sIgA) (**Figure 5**), i.e. dimeric IgA formed by two antibody monomers covalently bound through disulfide bonds to the J chain, plus a secretory component (SC) derived from the glycosylated transmembrane protein named pIgR (polymeric Ig receptor) **(Pabst and Slack, 2020)**. In mice a single IgA isotype is present, whereas humans have two isotypes, IgA1 and IgA2. IgAs produced by plasma cells in the mammary gland are transported across alveolar epithelial cells (ECs) by the pIgR; at the apical surface, proteolytic cleavage releases sIgA. In suckling infants, SIgA antibodies shape the composition of the gut microbiota and promote a mutualistic relationship with the host. Furthermore, coating of intestinal bacteria by sIgA is a fundamental mechanism to prevent bacterial translocation into the circulation and, thus, the colonization of distant organs. Clinically, this is reflected by lower incidence on neonatal sepsis in neonates fed with human breast milk compared to formula milk, as well as lower incidence and severity of necrotizing enterocolitis (NEC) **(El Manouni El Hassani et al., 2019; Patel and Kim, 2018)**. For what concerns their antigen specificity, secretory IgA can be antigen specific, i.e. secreted by mammary plasma cells through a T cell-dependent mechanism, or "natural", i.e. broadly reactive

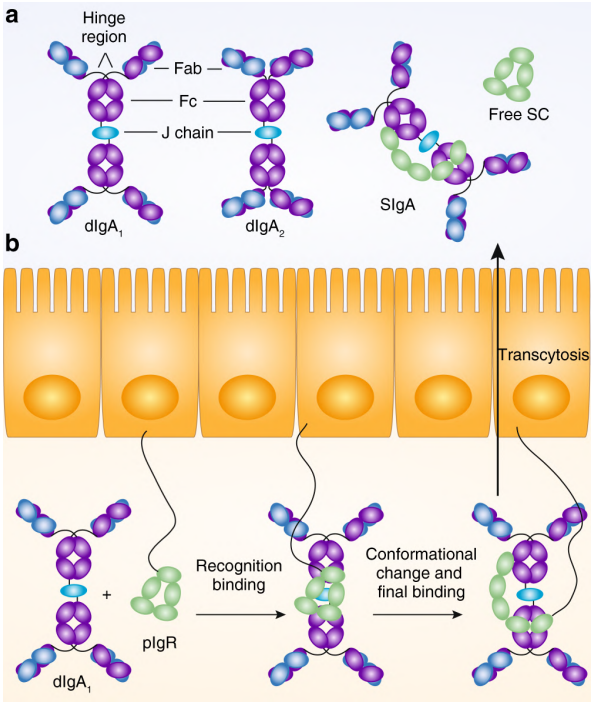

Figure 5. a) representation of human dimeric IgA_1, IgA_2, and secretory IgA, with free soluble compound (SC). b) interaction of dimeric IgA (dIgA) with polymeric Ig receptor (pIgR) to produce the final form of sIgA. Reproduced from Pabst O, Mucosal Immunol 2020 **(Pabst and Slack, 2020)**

and T cell- independent. The spectrum of antigen-specific sIgA provided by breast milk reflects that of maternal intestinal sIgA, as mammary gland plasma cells originate themselves from the mesenteric lymph nodes (MLNs) **(Roux et al., 1977)**. Nonetheless, as recently demonstrated, even natural sIgA can have a fundamental protective effect against bacterial translocation in the neonatal intestine **(Zheng et al., 2020)**. Finally, it's important to note that the combined transfer of members of the maternal microbiota with the corresponding, antigen-specific maternal sIgA might provide the optimal strategy to rapidly establish a stable host–microbial homeostasis in the neonatal gut.

1.3 Other factors affecting the maturation and function of immune system

1.3.1 prenatal inflammation and infections

Maternal infection, and the subsequent local or systemic inflammatory response, is not a rare event during pregnancy **(Pietrasanta et al., 2019)**. Despite any maternal inflammatory response may potentially have an effect on the fetus, the most common and impactful situation in pregnancy is intrauterine infection – inflammation (IUI), i.e. an inflammatory process, often due to a local infection, that can involve the chorioamniotic membranes (chorioamnionitis) and the umbilical cord (funisitis) **(Peng et al., 2017)**. In the first case, the inflammatory response is mainly of maternal origin, in the second the fetus is directly involved. Both types of IUI are a frequent cause of preterm birth, and the incidence of IUI increases with decreasing gestational age (GA). At 23-24 weeks of GA, up to 80% of preterm births is due to IUI, but also at term of gestation an infection or a non-infective inflammatory mechanism may drive the onset of labor **(Pugni et al., 2016; Romero et al.,**

2015). Moreover, IUI can be accompanied by the premature rupture of membranes (PROM), a consequence of the local inflammatory response, but also a cause, in turn, of enhanced risk for bacterial colonization of the uterine cavity by microorganisms ascending from the genital tract. In case of inflammation-mediated preterm birth, with or without PROM, the microorganisms more frequently isolated from the maternal genital tract are members of the *Mollicutes* class, typically *Ureaplasma* and *Mycoplasma*, while at term of gestation *Streptococcus agalactiae* (also known as group B Streptococcus, or GBS) and Gram-negative *Enterobacteriaceae*, such as *Escherichia coli*, prevail **(DiGiulio, 2012)**. With or without a direct fetal involvement in the inflammatory process, it is know that prenatal IUI has a tremendous impact on the developing fetus, with long term consequences extending beyond the occurrence of preterm birth. Indeed, both inflammatory cytokines / chemokines of fetal origin and those produced by the mother and crossing the placental barrier can modify the developing trajectories of most biological systems, including the respiratory tract **(Kallapur, 2006)**, the central nervous system **(Ylijoki et al., 2012)**, and the immune system **(Kallapur et al., 2014)**. For what concerns specifically the immune system, it has been noted that human neonates born to mothers with IUI present an increased number and activation of both neutrophils and monocytes at birth **(Jackson et al., 2017)**, high expression of S100A alarmin in cord blood monocytes **(Golubinskaya et al., 2020)**, a different polarization and transcriptome of $CD4^+$ T helper cells in the cord blood, with a predominance of Th17 polarization, an increased production of IL-17, and diminished Treg function compared to neonates not exposed to IUI **(Rito et al., 2017; Weitkamp et al., 2016)**. A similar phenotype has also been noted in experimental animal models of prenatal inflammation: for example it has been demonstrated that maternal IL-6 induced by systemic prenatal inflammation can have a tissue-specific effect on fetal and neonatal

immune cells of the mucosae, targeting in particular the small intestine immune system. In the SI, maternal IL-6 can directly impose epigenetic changes on epithelial stem cells, leading to long-lasting impacts on intestinal immune homeostasis, making the offspring less susceptible to postnatal intestinal infections but more prone to pure inflammatory states, such as colitis **(Lim et al., 2021)**. Thus, maternal infection can also promote long-term, tissue-specific fitness of the fetus.

1.3.2 prenatal and neonatal antibiotics

In the context of prenatal IUI or abnormal bacterial colonization of the maternal genital tract, the first-line medical intervention is the administration of antibiotic therapy to pregnant mothers before birth, and to neonates after birth. In humans, antibiotics are among the most frequently prescribed drugs during pregnancy, and according to recent data up to 25-35% of women receive at least one antibiotic prescription during pregnancy **(Bookstaver et al., 2015)**. The most commonly administered antibiotics are in the class of β-lactams (e.g. ampicillin and amoxicillin), which are variably used for PROM therapy before term, for urinary trait infections, and for peri-partum prophylaxis of GBS colonization, and in the class of macrolides (e.g. azythromycin, erythromycin), used for the combination therapy of PROM **(2020)**. Moreover, neonates whose mothers have received antibiotics during the last part of pregnancy are frequently treated with antibiotics themselves, because of an ongoing infection or an increased relative risk for infection. In this scenario, the most commonly prescribed molecules are again β-lactams, and aminoglycosides, such as neomycin and gentamycin **(Cotten et al., 2012; Shane et al., 2017)**. Thus, a large part of neonates, especially those born prematurely, is exposed to antibiotics during pregnancy, or is still exposed during the first days of life. Despite such an extensive use, little is yet known about the microbiome-mediated effect of prenatal antimicrobials on neonatal

immune system. In humans, it has been shown that prenatal antibiotics greatly alter the neonatal intestinal microbiome, decreasing the abundance of *Bacteriodetes* and *Bifidobacteria*, with a concurrent increase of *Proteobacteria* **(Dierikx et al., 2020)**. Animal models offered the opportunity to confirm this effect, and to investigate more clearly the effect of single molecules on the composition of intestinal microbiome, but with the unavoidable bias of modeling such an effect on a non-human organisms, with their own unique microbiome **(Kennedy et al., 2018)**. What is the final functional effect of these modifications on neonates, and especially on their immune function, is only partially understood. Scientists have shown, on murine models, that perinatal antibiotics may decrease the neutrophils number and predispose to late-onset neonatal sepsis (LOS), through a ILC3-G-CSF – mediated mechanism **(Deshmukh et al., 2014)** and, similarly, that they may predispose to neonatal pneumonia **(Gray et al., 2017)**. These results were not clearly confirmed in humans **(Reed et al., 2018)**, possibly because of the heterogeneity of antibiotic exposure in a real clinical context, compared to experimental setting, and because animal models of antimicrobial therapy are frequently "extreme" compared to human clinical settings, both in terms of molecules used and of timing of administration (i.e. broad drug cocktails administered for long periods of time, both before and after birth). Nonetheless, in humans it has been shown that early-life antibiotics are associated with reduced growth in boys during the first six years of life **(Uzan-Yulzari et al., 2021)**, but the mechanism has not been elucidated, and the antimicrobial exposure considered was that of the first days of life, with direct administration of antimicrobials to neonates. For what concerns the effect of prenatal antibiotics, to date results are scarce and conflicting.

1.3.3 a window of opportunity: postbiotics

As previously mentioned, one of the most efficient and studied tools to modify the composition of maternal and early-life microbiome, and thus its effect on the developing neonatal immune system, is the use of probiotics **(Hemarajata and Versalovic, 2013)**. Notwithstanding, the use of live microorganisms as therapeutic weapons in such a delicate phase of life as pregnancy and the neonatal period has raised several concerns: first, the administration of high amounts of live bacteria is not completely risk-free, and despite their generally low pathogenicity, cases of neonatal sepsis caused by probiotics have been repeatedly described **(Cavicchiolo et al., 2019; Costa et al., 2018)**. Second, several probiotic strains are highly antibiotic-resistant, and carry plasmidic resistance genes that can be transferred to the host microbiota **(Zheng et al., 2017)**. Third, it is frequently difficult to establish the correct dose and strain to use, because there is great variability between the studies. Considered that at least a proportion of the biological effect of probiotics is due to their metabolic bioproducts, an intriguing alternative to the use of live organisms can be the administration of the so called "postibiotics", i.e. preparations of metabolic products of the bacterial strain of interest **(Tsilingiri and Rescigno, 2013)**. Positive effects of the administration of postbiotics on the neonatal mucosal immune system have been shown, on animal models **(Gao et al., 2019; He et al., 2017; Morales-Ferré et al., 2021)**. Among these, an accelerated epithelial maturation that may lead to diminished bacterial translocation, an increase in the transcription of TJ proteins, a possible boost to the production of mucosal IgA and of antimicrobial proteins. Nonetheless, these supplementation protocols have never been applied in the context of antibiotic-induced dysbiosis, nor the administration to pregnant mothers have been investigated.

Chapter 2: Aim of the Project

2.1 Primary objectives of the research

- To investigate the effect of clinically relevant, prenatally restricted (i.e. administered only before birth) antibiotics (Abx) on the survival and immune response of neonates to late-onset neonatal sepsis (LOS), on a murine animal model
- To clarify which are the immune determinants of the aforementioned effect, investigating both the role of neonatal immune system and that of breast milk
- To investigate the role of maternal nutritional supplementation with a postbiotic, specifically a dried soluble product of *Lactobacillus paracasei CNCM I-5220* aerobic fermentation named immunoFOS® (iFOS), on the immune modifications induced by prenatal antibiotics

2.2 Secondary objectives of the research

- To clarify whether the effects of prenatal antibiotics therapy on the immune function of the offspring are long-lasting, i.e. persist beyond the neonatal period
- To investigate the effect of prenatal antibiotics on the composition of neonatal intestinal microbiome, at different time points after birth
- To investigate the effect of prenatal antibiotics on maternal and neonatal intestinal structure and health

Chapter 3: Materials and Methods

3.1 Mice and breeding

Female C57BL/6JOlaHsd mice, 8-12 weeks old, were purchased from Envigo RMS (Udine, Italy). Male C57Bl/6 mice for breedings, 8-12 weeks old, were purchased from Charles River Laboratories (Calco, Italy). To avoid the effect of specific-pathogen-free (SPF) conditions on animal immune system and microbiome **(Beura et al., 2016)**, mice were housed under conventional conditions at Humanitas Clinical and Research Center (Rozzano, Milan, Italy), 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.14/19 and 567/2019-PR) approved by the Italian Ministry of Health, and consistent with national (D.L. N. 26, G.U. March 4, 2014) and inter- national law and policies (EEC Council Directive 2010/63/EU). Breedings were established with a 1:1 scheme in the late afternoon, after synchronization of female estral 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.

3.2 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.

3.3 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* (1×10^9 CFU g−1, approximately 3×10^9 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 **(Brook et al., 2019)**. 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 (not 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.

3.4 Construction of *E. coli* mCherry K1

E. coli K1 was transformed by electroporation using pONmCherry plasmid, a gift from Howard Shuman (Addgene plasmid # 84821) **(Gebhardt et al., 2017)**. 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 MgCl2 (1M, 10 µl/ml) and Glucose (1M, 20 µl/ml) 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.

3.4 Immunoglobulins quantification

Feces and stomach content of the pups (as a proxy of maternal breastmilk) were immediately weighted and frozen in dry ice 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.

3.5 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 NH4Cl 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.

3.6 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 **Table 1** for 30 minutes at 4°C in FACS buffer. Dead cells were excluded by using Fixable Viability Dye eFluor® 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).

Table 1. List of antibodies and fluorochromes used for flow cytometry and confocal microscopy

Antigen	Fluorophore	Clone	Cat.number	Supplier	Dilution
IgA	PE	mA-6E1	12-4204-8	Ebioscience	1:100
IgA	Unconjugated	polyclonal	1040-01	Southern Biotech	1:400
CD16/32	Unconjugated	93	14-0161-82	Ebioscience	1:50
CD45.1	FITC	A20	110706	Biolegend	1:100
CD45.1	APC-Cy7	A20	110716	Biolegend	1:100
CD45.2	FITC	104	11045485	Invitrogen	1:100
CD45.2	APC-Cy7	104	109824	Biolegend	1:100
CD3	PE-Cy7	145-2C11	552774	BD	1:200
CD4	PE	RM4-5	553048	BD	1:200
CD8	APC	53-6.7	553035	BD	1:100
TCRβ	FITC	H57-597	553171	BD	1:100
FoxP3	APC	FJK-16S	17-5773-82	Ebioscience	1:100
RORγt	PE	AFKJS-9	12-6988-82	Ebioscience	1:100
Ly6G	BV510	1A8	127633	Biolegend	1:200
Ly6C	BV605	AL-21	563011	BD	1:200
CD11b	BV421	M1/70	562605	BD	1:200

CD11b	Pe-Cy7	M1/70	552850	BD	1:200
CD103	PE	2E7	121406	Biolegend	1:100
CD11c	PerCP-Cy5.5	N418	35011482	Ebioscience	1:200
CX3CR1	AF647	SA011F11	149004	Biolegend	1:100
MHC-II	BV421	M5/114.15.2	562564	BD	1:100
CD138	PerCP-Cy5.5	281-2	142509	Biolegend	1:100
Fix.viability stain	eFluor 780	n.a.	65-0865-14	eBioscience	1:1000

3.7 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 **(Palm et al., 2014)**. 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.

3.8 Immunofluorescence

After animal euthanasia, pieces murine tissues were fixed overnight in "PLP" buffer (1% paraformaldehyde, L-Lysine 0.2M pH 7.4 and 25 mg 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 counter- stained 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.

3.9 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.

3.10 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).

3.11 Bacterial DNA extraction from fecal samples and quality control

DNA from fecal pellets was extracted with G'NOME DNA isolation kit (MP) following a published protocol (Furet et al., 2009). 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. 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.

3.12 Analysis of the microbiota composition by 16S rRNA gene sequencing

DNA quantity and integrity were checked through TapeStation 4200 (Agilent Technologies) and 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).

3.13 Generation and administration of diets supplemented with immunoFOS®

For experiments involving the administration of postbiotic-supplemented diet to pregnant dams, a dried soluble product of *Lactobacillus paracasei CNCM I-5220* aerobic fermentation was prepared and added to the diet as described below.

The fermentation of *Lactobacillus paracasei CNCM I-5220* was obtained with a two-step process: a first aerobic fermentation, starting after 7 hours of bacterial growth in culture medium, was carried on for approximately 23 hours in a 1000-liter industrial fermenter, monitoring temperature, pH, and agitation by vibromixing. At the end of the process, the bacterial culture was centrifuged, the culture medium discarded and the biomass obtained from

the first fermentation was transferred in a "minimal buffer solution" in the presence of short chain fructooligosaccharides (scFOS) and left to ferment for 24 hours. After the second fermentation, the culture was centrifuged to eliminate the bacteria and the supernatant, named "ImmunoFOS®" (iFOS), was dry sprayed after the addition of maltodextrin (MDX) as lyoprotectant. A control dry-sprayed powder was prepared with MDX only. The whole industrial protect is protected by patent #WO2019149941A1, under which full details of the iFOS production process can be found.

A treatment (supplemented with iFOS) and a control (supplemented with MDX) diet were then prepared. Briefly, standard chow diet was crumbled under sterile condition and re-pelleted adding 675 mg of sterile (0.2 μm filtered) iFOS of MDX, resuspended in sterile H_2O, per kilogram of diet. Considering an average food intake per dam of 6 g/day, an average daily dose of 135mg/kg of iFOS or control MDX was administered to each pregnant animal, starting on E13.5 and ending on E18.5 or P14, depending on the experiment. This route of administration was preferred to a daily gavage of PB600F/MDX solution to avoid the gavage-related stress during the last week of pregnancy. After E18.5 or P14, pregnant and lactating females were returned to a standard diet.

3.14 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. 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.

Chapter 4: Results

4.1 Effect of prenatal antibiotics on maternal gut

To mimic the exposure of pregnant women to antimicrobials during the last trimester of pregnancy, as it can happen in case of increased risk for preterm delivery or of overt intrauterine infection-inflammation (IUI), we administered to pregnant C57Bl/6 dams a mixture of three antibiotics ("Abx": ampicillin, a broad spectrum penicillin, + neomycin, an aminoglycoside, + erythromycin, a macrolide) in drinking water, or normal drinking water as control, between embryo-day 13.5 (E13.5) and E18.5. The general experimental model is represented in **Figure 6**.

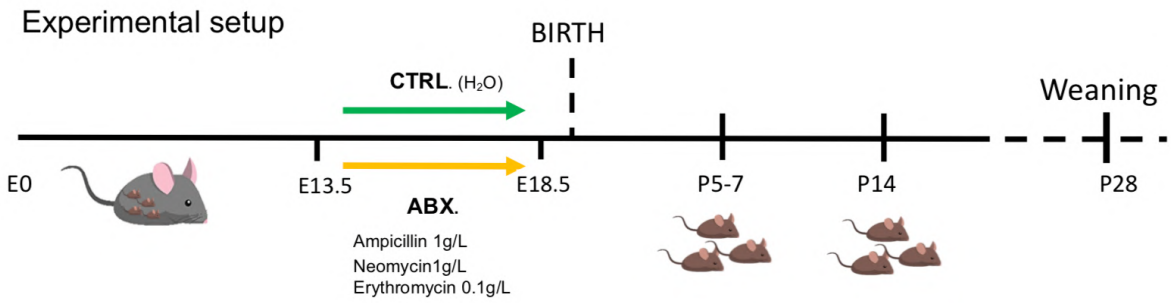

Figure 6. Schematic representation of the general experimental setup

Prenatal Abx caused the rapid establishment of a clear dysbiotic status in the mothers, as highlighted by a significant reduction in the bacterial load of fecal pellets, both aerobes and anaerobes, already after the first 36 hours of administration (**Figure 7A**). Furthermore, within 5 days of treatment, prenatal Abx induced a clearly visible enlargement of the cecum, filled with intestinal mucus, and the elongation of small intestine, while colon length was not affected (**Figure 7 B-C**). Abx-induced dysbiosis was also matched by a reduction in the transcription of several antimicrobial proteins and peptides by the small intestine wall, such as Regenerating islet-derived protein 3

gamma (Reg3γ) and defensin alpha-5 (Defα–5), and by an almost significant increase in the transcription of the most abundant mucin gene, MUC2 (**Figure 7D**).

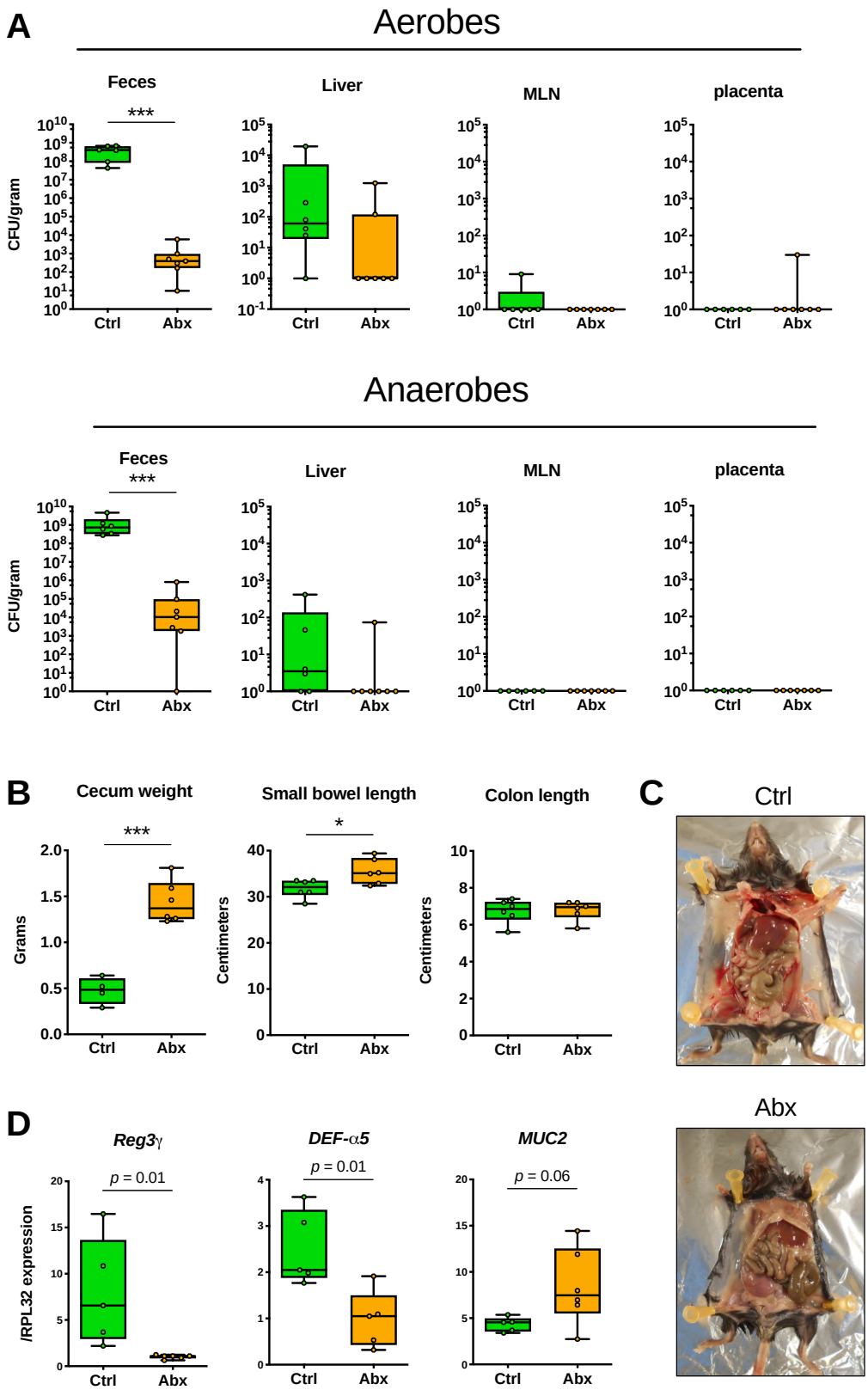

Figure 7. (A) amount of culturable aerobic and anaerobic CFUs in maternal organs after 5 days of treatment with antibiotic (Abx) or vehicle (Ctrl). (B) anatomical parameters in mothers treated or non-treated with antibiotics during late pregnancy. (C) Visual appearence of small and large bowel without 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

Despite these clear functional modifications, neither epithelial structure of maternal small intestine nor the most important protein of the endothelial gut-vascular barrier (GVB) **(Spadoni et al., 2015)**, the plasmalemma vesicle-associated protein (PLVAP) were affected by Abx treatment: indeed, both confocal microscopy imaging analysis (**Figure 8 A-B**) and the quantification of mRNA levels (**Figure 8C**) did not show clear alterations of epithelial tight junctions (TJ) – associated proteins (Zonula occludens-1, ZO-1, occludin) or GVB. Accordingly, when we took advantage of the FITC-dextran functional assay to evaluate the intestinal permeability to small (4 kDa) fluorescent molecules, we did not detect a clear alteration in the plasma concentration of FITC-dextran after Abx treatment (**Figure 8D**).

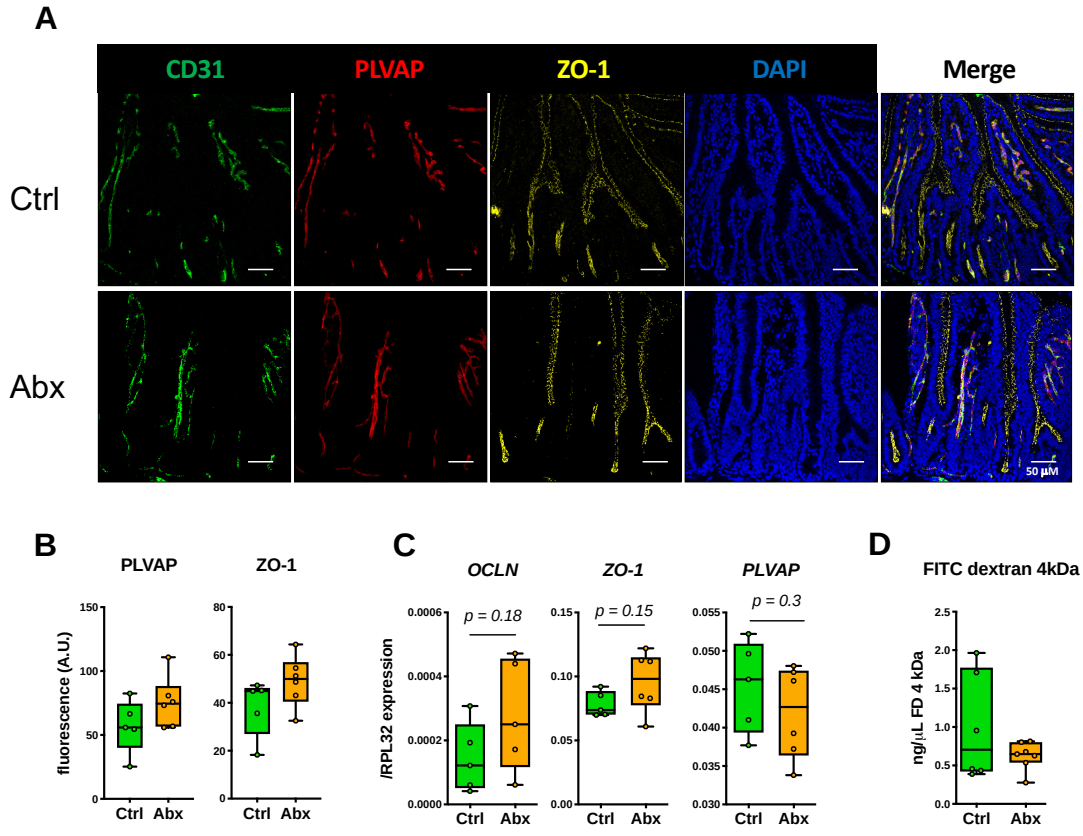

Figure 8. (A) Confocal microscopy images of plasmalemma vesicle-associated protein (PLVAP) and zonula occludens.-1 (ZO-1) proteins in maternal ileum at E18.5, with or without Abx treatment. (B) Quantification of PLVAP and ZO-1 fluorescence in (A). (C) Gene expression (relative to housekeeping RPL32) of tight junction proteins (OCLN, ZO-1) and PLVAP in maternal ileum. (D) 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

Thus, we conclude that maternal antibiotic treatment during the last 5 days of pregnancy in C57Bl/6 mice profoundly alters the microbial content of intestine and the excretion of antimicrobial proteins and peptides in the lumen, without significantly affecting the structure of intestinal epithelium, of thr GVB, and its permeability.

4.2 Effect of prenatal antibiotics on neonatal systemic immunity and intestinal structure

Experimental evidences from published scientific literature suggest that the exposure of neonatal mice to antimicrobials in early life can alter several aspects of immune function. Nonetheless, most experimental models apply prolonged antibiotic exposure both before and after birth, and/or non clinically relevant antibiotic cocktails, frequently including the oral administration of vancomycin. With our experimental model of clinically relevant antibiotics strictly administered only before birth, with no direct postpartum exposure of the pups, we detected no significant alteration at 5 days after birth (P5) in immune populations of blood ($CD45^+$ $CD11b^+$ $Ly6C^{int}$ $Ly6G^+$ neutrophils, $CD45^+$ $CD11b^+$ $Ly6C^{low}$ $Ly6G^-$ resting monocytes and $CD45^+$ $CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$ activated monocytes) or spleen (neutrophils, resting and activated monocytes, $CD45^+$ $CD11b^-$ $CD3^+$ $CD4^+$ and $CD8^+$ lymphocytes, **Figure 9A-C**). Thus, differently from what has been reported by others **(Brook et al., 2020; Deshmukh et al., 2014)**, prenatal, short-term Abx treatment of pregnant mothers does not seem a sufficient to induce quantitative modifications of the systemic immune compartment in the offspring during early life.

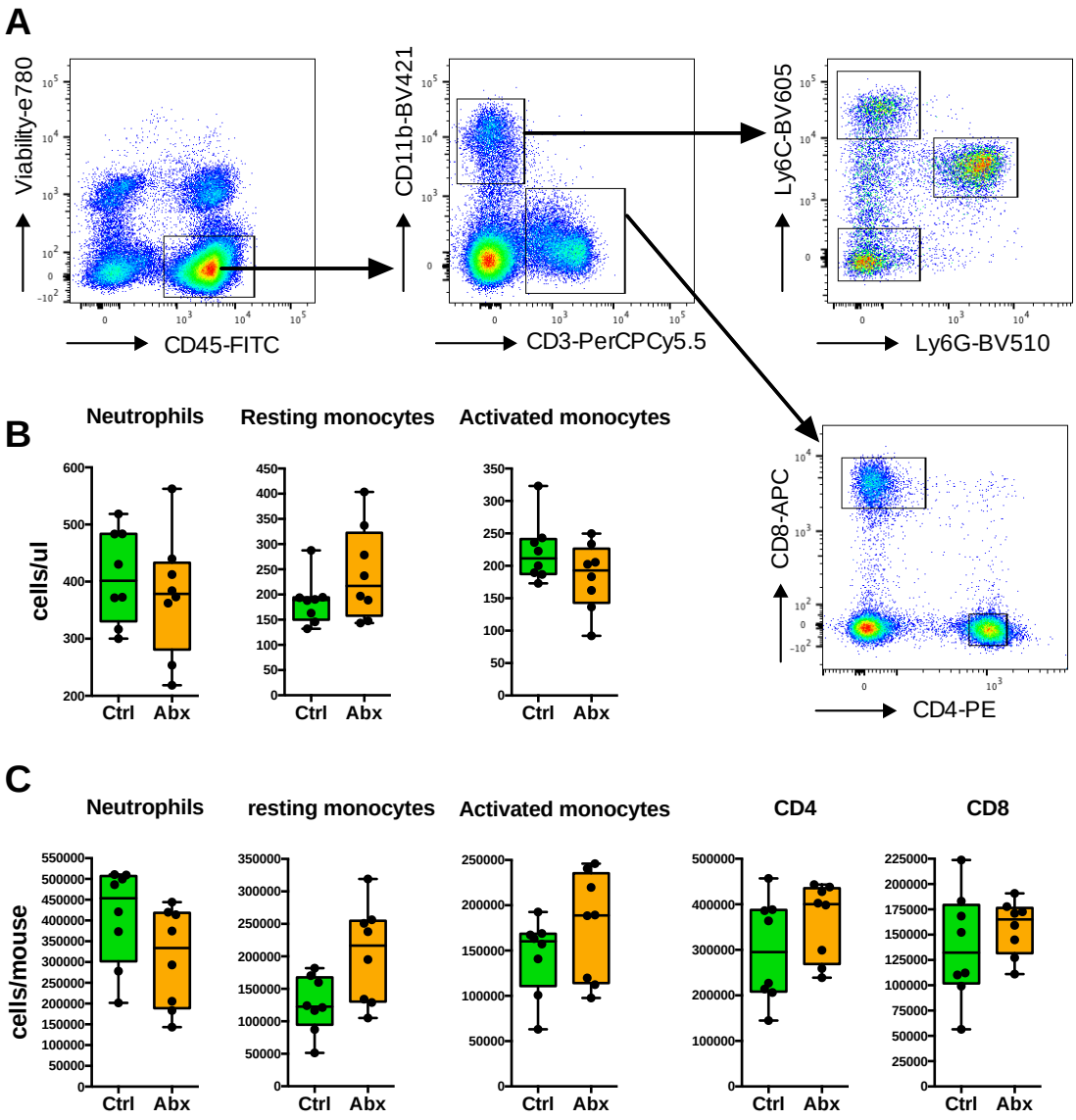

Figure 9. (A) Gating strategy used for both blood and spleen to identify myeloid and lymphoid populations. (B) Absolute amount of circulating myeloid cells at P5 in pups born to Abx-treated or untreated mothers. (C) Absolute amount of spleen myeloid and lymphoid cells at P5 in pups born to Abx-treated or untreated mothers. N= 5-8 mice per group, representative of 2 independent experiments. *P*-values calculated by t-test.

We then investigated whether the exposure to prenatal Abx, and the consequent dysbiosis, could alter the intestinal structure and permeability. By means of confocal fluorescence microscopy and mRNA quantification at P5, we detected no significant difference between pups exposed or not exposed to prenatal Abx in the expression of epithelial TJ proteins (ZO-1 and Claudin 3),

in the production of mucus proteins (Mucin 2, MUC2) and in the amount of fluorescent dextran (4 kDa FITC-dextran) retrievable from serum 4 hours after gavage (**Figure 10, A-C**). These results, even more in light of pre-existing literature, underlined how immune alterations induced by Abx-dependent dysbiosis are strictly related to the experimental conditions applied, and possibly less obvious in case of more clinically relevant experimental models.

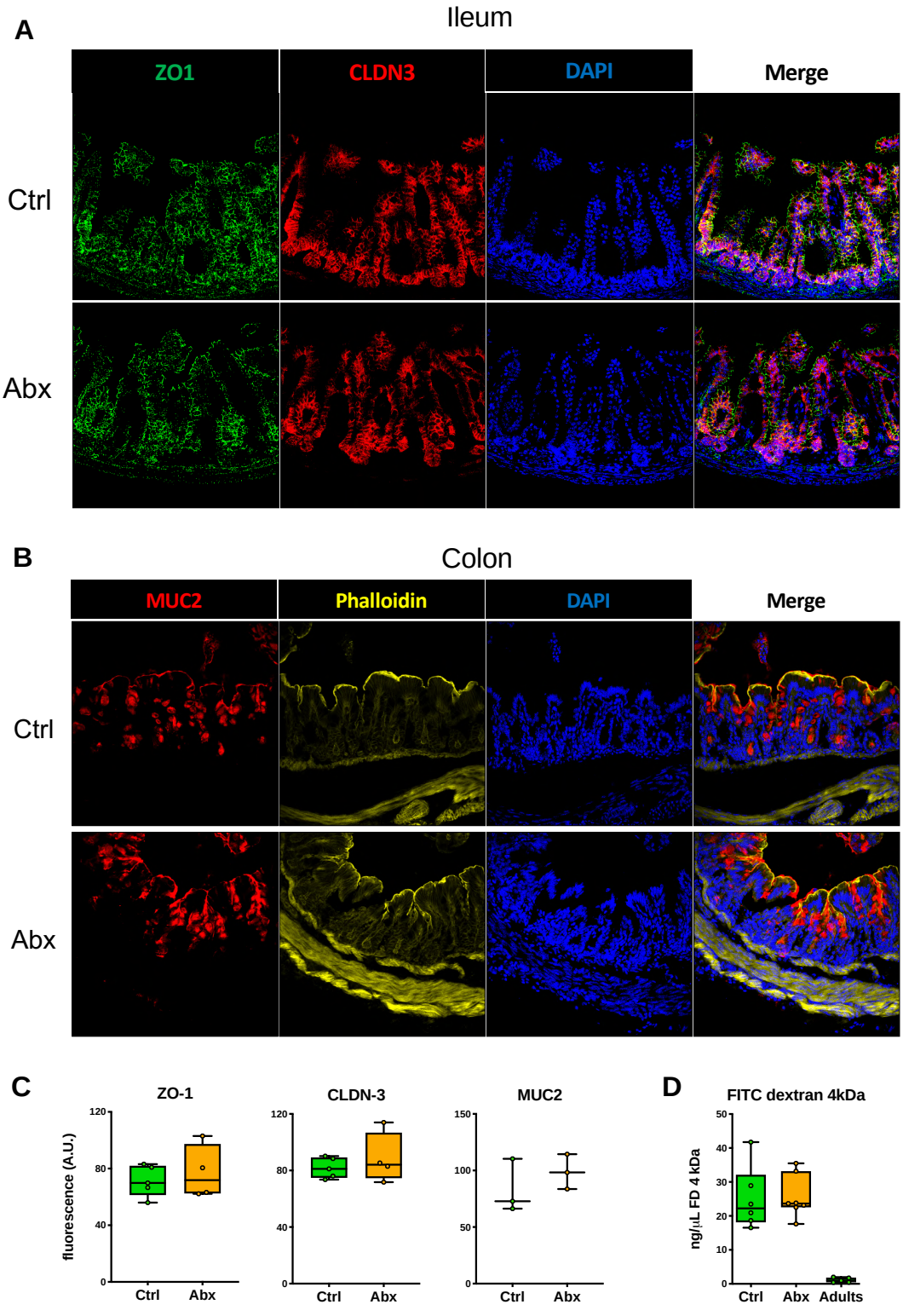

Figure 10. (A) Confocal microscopy images of neonatal ileum at P7 without or with prenatal Abx treatment. ZO-1: zonula occludens-1. CLDN-3: claudin 3. (B) Confocal microscopy images of neonatal colon at P7 without or with prenatal Abx treatment. MUC2: mucin-2. (C) Quantification of fluorescence in A (ZO-1, CLDN-3) and B (MUC2). (D) Serum concentration

of 4 kDa FITC dextran 4 hours after gavage in pups at P7. N= 5-8 mice per group, representative of 2 independent experiments. *P*-values calculated by t-test.

4.3 Effect of prenatal antibiotics on the response to a late-onset sepsis model

Despite the first results obtained, we were aware that the variables analyzed represented only few, individual aspects of a complex pool of biological mechanisms induced by maternally administered Abx and the subsequent systemic dysbiosis. To gain a more direct, comprehensive and functional insight into the consequences of prenatal Abx therapy on neonatal immune response, we decided to investigate the susceptibility of pups either exposed or not exposed to prenatal Abx to an experimental model of neonatal late-onset sepsis (LOS), challenging 4-5 days old pups (P4-P5) with a high dose of live *Escherichia coli serotype O18:K1* administered by gastric gavage. This experimental model of sepsis presents several advantages over others, such as:

1. It mimics the human neonatal LOS caused by translocation of intestinal bacterial from the gastrointestinal lumen from the bloodstream
2. *Escherichia coli serotype O18:K1* is a frequent human neonatal pathogen, and the main cause of Gram- neonatal meningitis occurring after intestinal translocation
3. The use of a live pathogenic bacterium allows to evaluate a more clinically relevant scenario compared to the use of dead bacteria or bacterial components, such as lipopolysaccharides (LPS)
4. Differently from direct systemic injection (e.g. intraperitoneal) of pathogens or LPS, gavage models allows to evaluate the barrier

function of intestinal epithelium, the importance of gastrointestinal immune system, and the possible interaction in the lumen between the invading pathogen and other component of the host microbiome

For this first functional experiment, we decided to include a third group of mice exposed to Abx both prenatally and postnatally (**Figure 11A**), up to 24 hours before the bacterial challenge, to have a "reference" group consistent with previously published literature **(Deshmukh et al., 2014; Singer et al., 2019)**. Pups born to mothers not exposed to Abx during pregnancy (**Figure 11B**, 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). As expected from previously published literature, pups challenged with *Escherichia coli serotype O18:K1* after exposure of pre-postnatal Abx (**Figure 11B**, red line) were significantly more susceptible to LOS, with a median survival of 2.75 days. Surprisingly, the survival curve of pups exposed to Abxs only during the fetal life (**Figure 11B**, yellow line) closely resembled that of pups with prolonged exposure, with a median survival of 2.5 days and a mortality rate up to 63%. This significantly increased susceptibility correlated with an increased translocation of *E.coli* to distant organs (**Figure 11C**): indeed, mesenteric lymph nodes, liver, and spleen of infected newborn mice of the Abx group showed a significantly higher bacterial load 48 hours after infection, as compared to infected control pups.

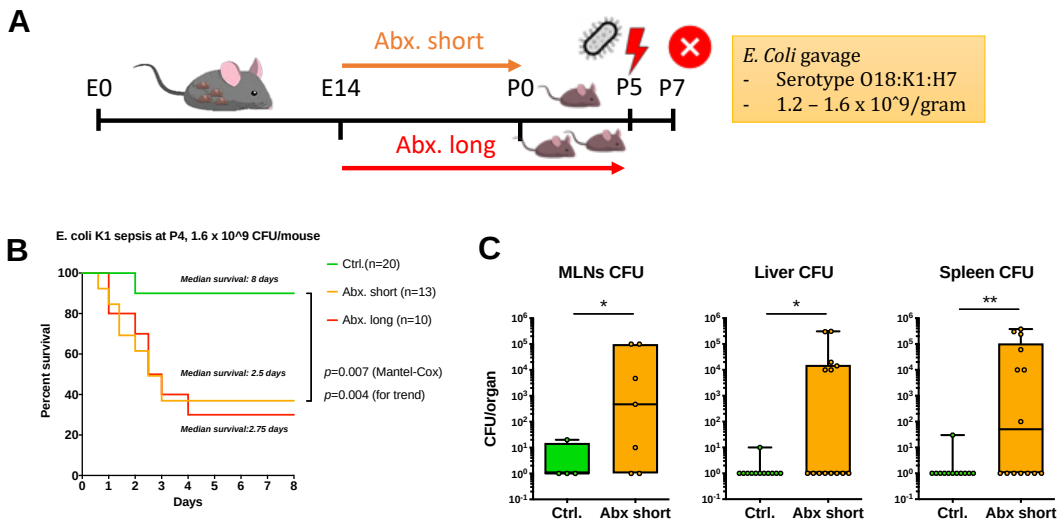

Figure 11. (A) Schematic representation of experimental procedure used for LOS experiment. (B) Survival curves of mice after LOS induced by *E.coli K1*. Pooled results from 3 independent experiments. (C) Quantification of *E.coli* colonies (colony-forming units, CFU) 48 hours after gavage in peripheral organs. MLNs: mesenteric lymph nodes. N= 5-8 mice per group, representative of at least 2 independent experiments. *P*-values calculated by t-test. *<0.05, **< 0.01

4.4 Prenatal antibiotic exposure and 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 functional aspects of the intestinal immune system. It is known that luminal antibodies, in particular the predominant subclass of secretory IgA (sIgA), coat and contain the commensal and pathogenic microbiome, and that in early life maternal breastmilk is the most important source of sIgA for the neonatal gut. In turn, robust experimental evidences have shown that commensal microbiome is able to stimulate and shape the production of sIgA at the mucosal sites, in a reciprocal feedback loop. Therefore, we reasoned that prenatal Abx treatment and the consequent

dysbiosis, both in the mother and in the neonates, may 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 i*n utero* (1173 ± 432 vs 358 ± 379 μg/g, $p < 0.01$, **Figure 12A**). The functional counterpart of this quantitative reduction was a lower proportion of fecal bacteria coated by the same IgA, as assessed by flow cytometry, in pups born to dams exposed to Abx (**Figure 12B-C**). Considered, as assessed above, that breast milk is the major source of sIgA during breastfeeding, 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 (77 ± 31 vs 47 ± 29 μg/mL, $p < 0.05$, **Figure 12BD**). To confirm these results, we also analyzed the mammary glands of mothers exposed or not exposed to Abx by means of flow cytometry and confocal fluorescent microscopy. As highlighted in **Figure 12E-F**, $CD45^+$ IgA^+ mammary plasma cells were still significantly reduced one week after the interruption of Abx, supporting the evidence that maternal intestinal dysbiosis caused by Abx is reflected by a reduction of breastmilk sIgA and, ultimately, by a reduced ability of IgA to coat microbiota in the gut of neonatal mice.

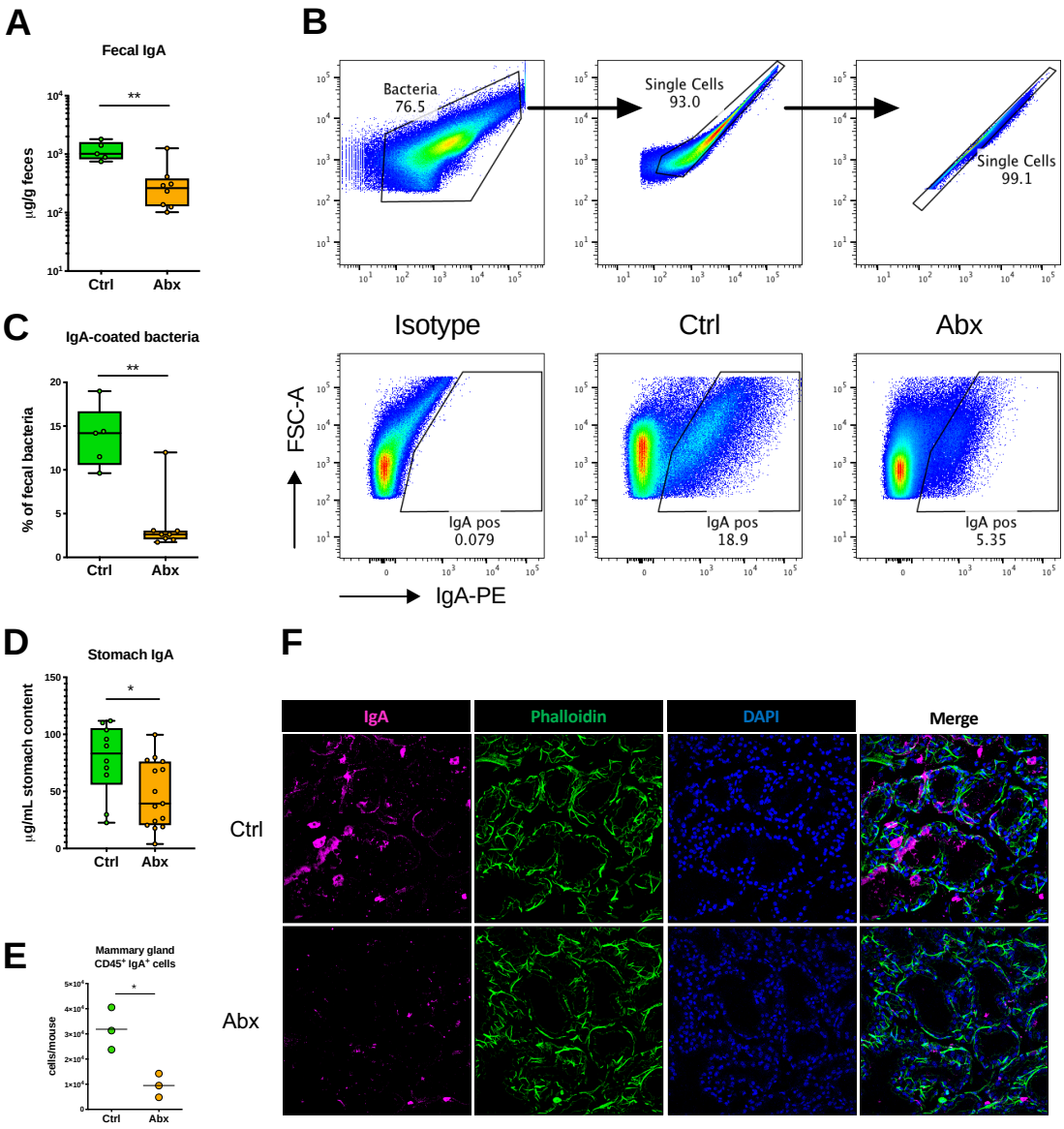

Figure 12. (A) Quantification of fecal IgA at P7 in pups born to untreated or abx-treated mothers. (B) Gating strategy used to identify IgA-coated bacteria in fecal pellets. (C) Percentage of fecal bacteria coated by IgAs in the two experimental groups. (D) Quantification of stomach IgA at P7 in the two experimental groups. (E) Absolute number of $CD45^{+}$ IgA^{+} live cells in the mammary gland of abx-untreated or treated dams at P7. (F) Confocal microscopy images of the mammary gland of abx-untreated or treated dams at P7. N= 5-8 mice per group, representative of at least 2 independent experiments for A, C, D. N= 3 mice per group, representative of 2 independent experiments for E. *P*-values calculated by t-test. *<0.05, **< 0.01

4.5 sIgA coating of pathogenic *E.coli*

To gain insight into the functional consequences of reduced IgA in our experimental LOS model, we constructed a fluorescent *E.coli* transformed by electroporation using pONmCherry plasmid, making the bacterium fluorescent to light in the wavelength between 540-590 nm. We then performed the LOS experiment, confirming that a significantly lower proportion of *E.coli* was coated by sIgA (**Figure 13A-C**).

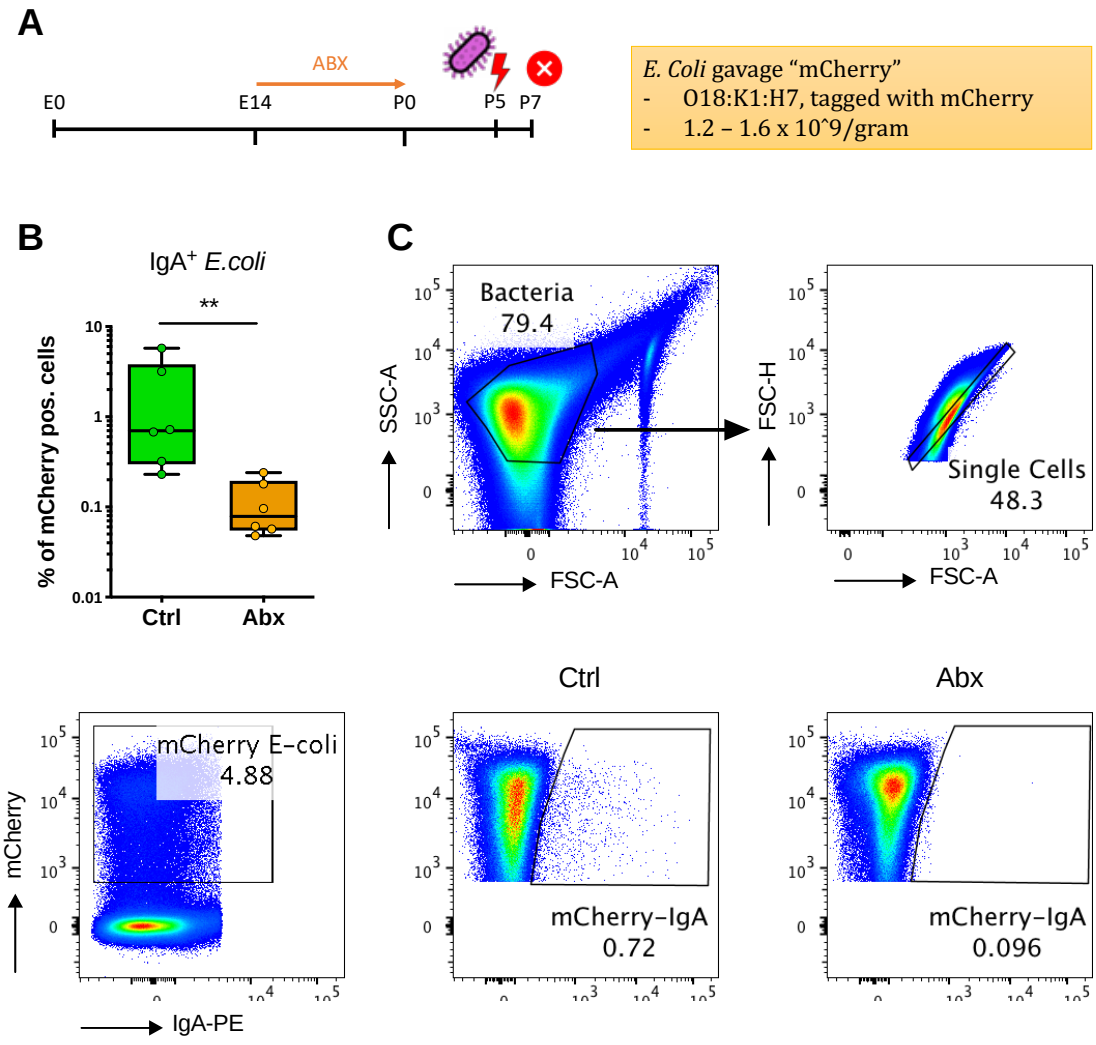

Figure 13. (A) Schematic representation of experimental procedure used for LOS experiment. (B) Quantification of the IgA-coating of fluorescent *E.coli K1* in fecal pellets 24 hours after gavage. (C) Gating strategy used to identify IgA-coated fluorescent *E.coli*. *N*= 6 mice per group, representative of 2 independent experiments. *P*-values calculated by t-test, **< 0.01

Thus, coating by IgA in the gastrointestinal lumen is critical for the protection against pathogen translocation in the context of neonatal LOS, and is reduced after maternal exposure to Abx during pregnancy.

4.6 long-lasting effect of prenatal antibiotics on intestinal IgA

Early life is a critical phase of development for the majority of biological functions, including those of the immune system. Any perturbation to the physiological establishment of immune functions, as that induced by Abx, can have an impact that extends beyond the strict neonatal period, as it has been repeatedly shown in humans and in experimental animal models **(Belkaid and Hand, 2014; Uzan-Yulzari et al., 2021)**. In humans, breastfeeding is progressively integrated by solid diet during the first year of life, and the maturation of gastrointestinal immune system is supported and stimulated by the encounter with food antigens. In mice, the same biological events occur over a period of a month, offering a window of opportunity to investigate the progressive maturation of immune function. In particular, breastfeeding is exclusive during the first 9-12 days of life, integrated by solid food between 12-16 days of life and progressively abandoned during the third week of life. By 28 days of life, young mice eat exclusively solid food, and mothers do not produce breastmilk anymore.

In our experimental model, the absolute amount of fecal IgA in mice exposed to prenatal Abx remained significantly lower, as compared to not exposed pups, at 14 days of life (P14), and trended towards a lower amount even at 28 days of life (**Figure 14A**). Similarly, the amount of stomach IgA was also reduced at P14 (**Figure 14B**), indicating that breastmilk was still representing a significant source of luminal IgA for the pups. Interesting, 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 exposed to prenatal Abx (**Figure 14C**). We reasoned that this difference may be explained by the different kinetics of bacterial colonization in pups of Abx-treated or untreated mothers: 16s rRNA sequencing of fecal bacteria, which is ongoing at present, will help to clarify these results.

It is known that most luminal sIgA within the gastrointestinal tract of adult mice are produced in the ileum, by $CD45^+ CD19^- CD38^+$ plasma cells residing in the LP and in Peyer's patches (PP) **(Fagarasan et al., 2010)**. Our next aim was then to quantify by means of multiparameter flow cytometry and confocal microscopy the IgA-producing plasma cells in the ileum lamina propria of neonatal mice at different postnatal ages, to evaluate their contribution to the total pool of luminal sIgA, and the influence of prenatal Abx on their development. Among $CD45^+$ IgA^+ plasma cells, we evaluated separately the two subsets of $CD11b^+$ and $CD11b^-$ plasma cells, as they can have different functional correlation with the intestinal microbiome **(Kunisawa et al., 2013)**. In Abx unexposed pups, IgA^+ plasma cells in the ileum LP at P7 were very limited, composing less than 1% of $CD45^+$ single cells (**Figure 14D-E**). At P14, with the exposure to the first solid food antigens, the proportion of IgA^+ cells increased significantly compared to P7, with a significant, although temporary wave of $CD45^+$ IgA^+ $CD11b^+$ cells. Then, at the age of P28, the proportion of IgA^+ LP plasma cells was similar to that of adult mice, with a clear prevalence of $CD11b^-$ cells. When compared with control mice, pups exposed to prenatal Abx had lower amounts of total $CD45^+$ IgA^+ plasma cells at both P14 and P28, with a predominant reduction in $CD11b^+$ cells at P14 and of $CD11b^-$ cells at P28. The quantitative evaluation obtained with flow cytometry at P28 was also visually confirmed with confocal microscopy at P28 (**Figure 14F-G**). By means of FACS, we also quantified the amount of $CD11b^+$ $CD11c^+$ $CD103^+$ dendritic cells (DCs) and of $CD11b^+$

$CX3CR1^+$ $CD11c^-$ mucosal macrophages (MΦs) at different postnatal ages in pups exposed or not exposed to prenatal Abx (**Figure 14H-I**). Both at P14 and at P28, pups born to Abx-treated dams had lower amounts of intestinal DCs, and lower amounts of intestinal MΦs at P28 as well.

These findings collectively suggested that the dysbiosis induced by prenatal Abx can have long-lasting effects on the pool of intestinal sIgA, due to a combination of reduced IgA introduced through breastmilk in the first days of life and an impaired maturation of the DCs - MΦs – plasma cells axis in the neonatal ileal LP.

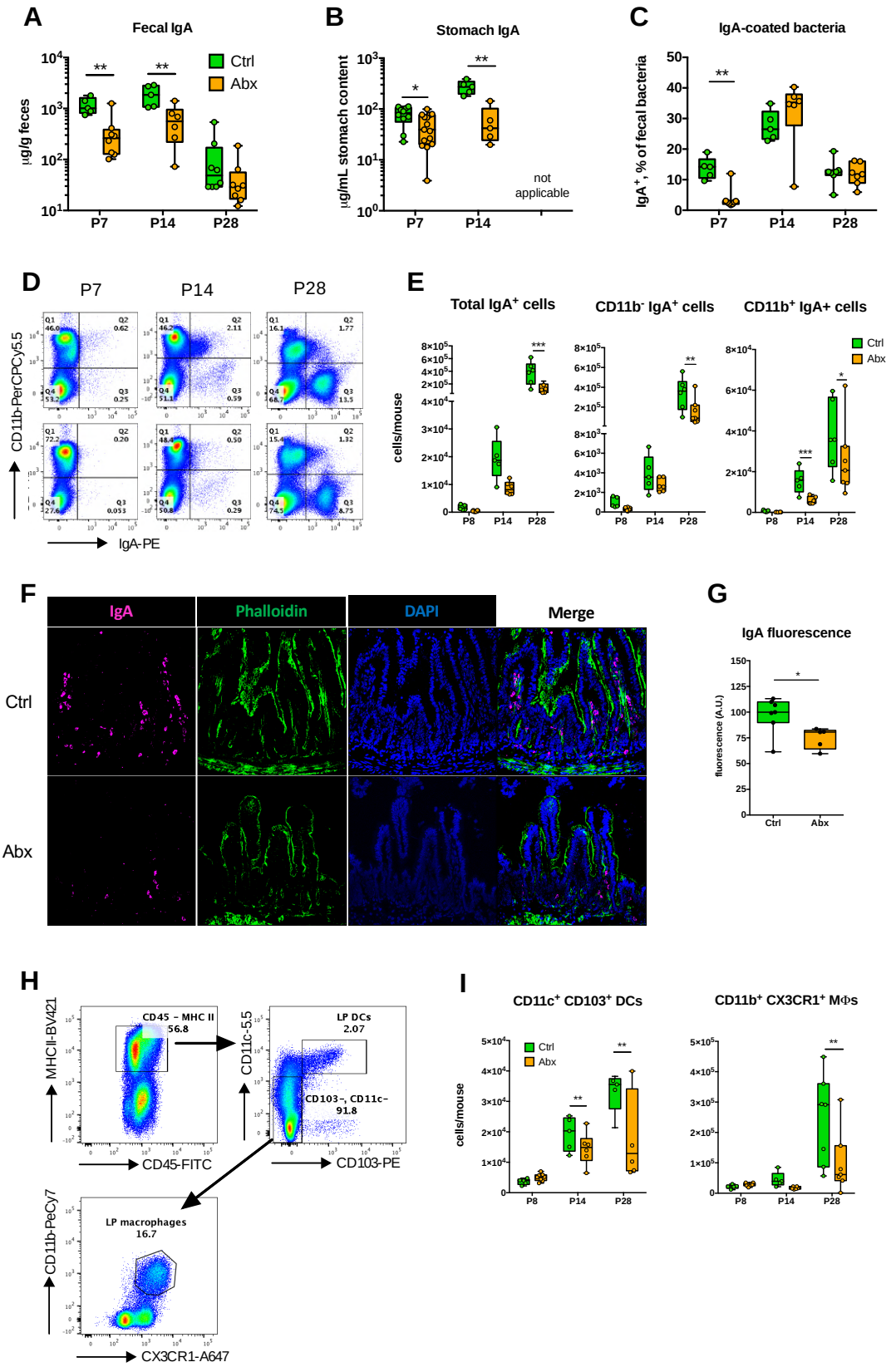

Figure 14. Quantification of fecal IgA (A), stomach IgA (B) and IgA-coating of fecal bacteria (C) at different postnatal ages in pups born to untreated (Ctrl) or abx-treated (Abx) dams. N= 6-15 mice per group, representative of at least 2 independent experiments. *P*-values

calculated by two-way ANOVA with Sidak correction for multiple comparisons. *<0.05, **< 0.01. (D) Flow cytometry panels of CD11b-positive or -negative plasma cells in the lamina propria (LP) of neonatal ileum, at different postnatal ages, in the two experimental groups. (E) Quantification of total IgA^+ plasma cells and of $CD11b^{+/-}$ subpopulations of plasma cells at different postnatal 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. *<0.05, **< 0.01, ***<0.001. (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, *<0.05 (H) gating strategy used to identify dendritic cells (DCs) and macrophages (MΦs) in the ileum LP of pups born to Abx untreated or treated mothers. (I) quantification of total DCs and MΦs in the LP at different postnatal 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. *<0.05, **< 0.01, ***<0.001.

4.7 Effect of prenatal antibiotics on intestinal T-helper cells

Very recently, it has been shown that both bacterial colonization of germ-free dams during pregnancy, and maternal infection of normally colonized females, can alter the lymphoid compartment of intestinal immune system in the offspring. In particular, maternal colonization/infection with Gram-intestinal bacteria increases the number of $CD4^+ ROR\gamma T^+$ T lymphocytes **(Lim et al., 2021)**, also known as T-helper-17 cells because of their ability to produce IL-17, as well as the number of innate lymphoid cells type 3 (ILC3: $lineage^- CD4^+ ROR\gamma T^+$), i.e. the innate, non-antigen-specific counterpart of Th17 **(Gomez de Agüero et al., 2016)**. Both these cell types are important in the maturation of mucosal lymphoid organs (they are also known as lymphoid tissue inducers, or LTi), and they participate in effective antimicrobial defense through the production of IL-17 and IL-22 **(Eberl et al., 2015)**. Considered these and our previous results, we hypothesized that maternal Abx-induced dysbiosis during pregnancy could also modify these cell populations in the offspring. As shown in **Figure 15**, prenatal Abx treatment marginally affected the number and proportion of $ROR\gamma T^+$ ILC3 in both ileum and colon, but significantly reduced the absolute number and the

percentage (among $CD4^+$ T cells) of Th17 cells, in ileum as well as in colonic lamina propria, and the amount of colonic T-reg cells (**Figure 15C-D**). Thus, prenatal Abx treatment of pregnant mothers can also negatively affect the lymphoid compartment of gastrointestinal mucosal immune system.

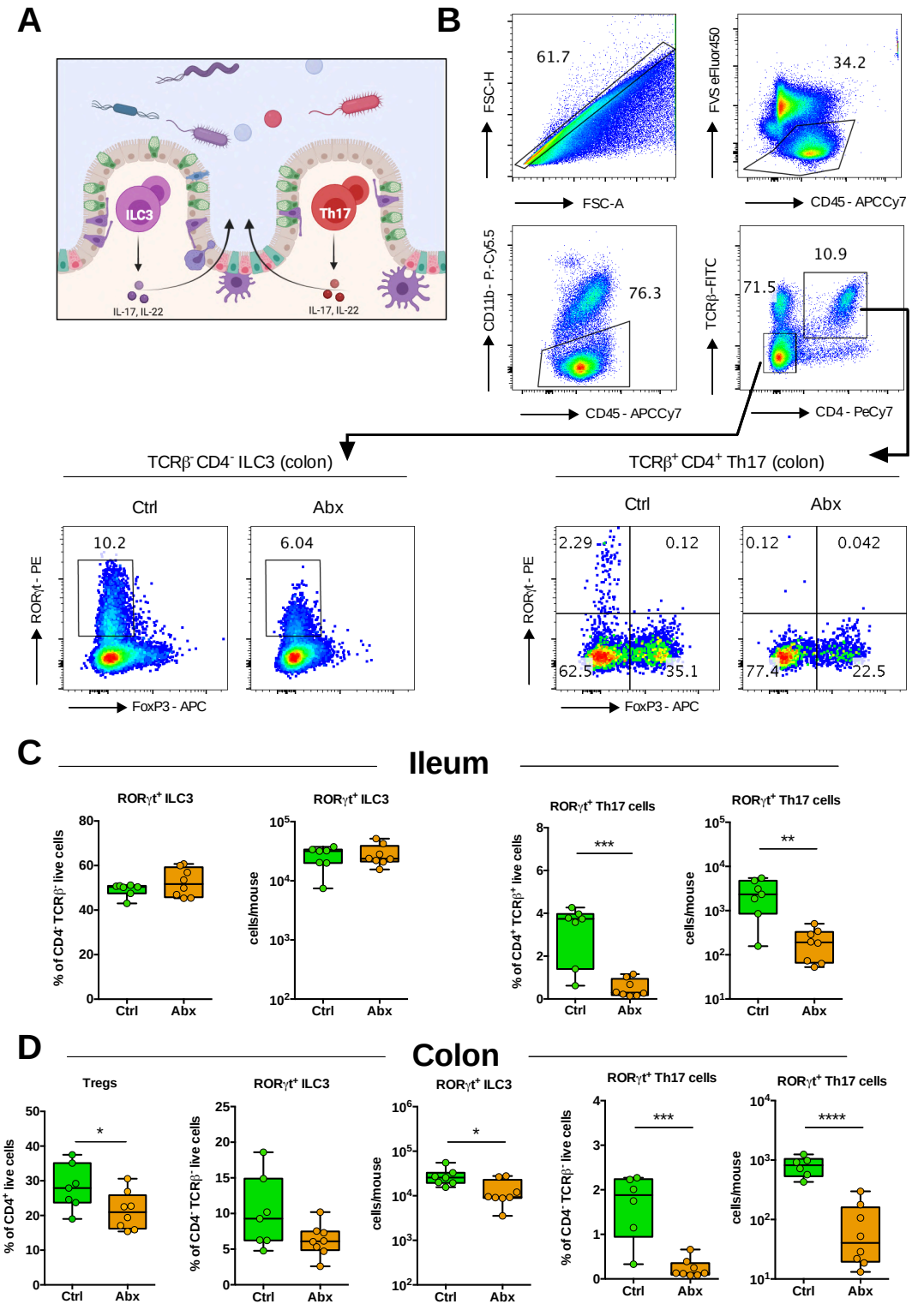

Figure 15. (A) Representation of Th17 and ILC3 populations in the intestinal mucosa. (B) Gating strategy used to identify ILC3 (CD45⁺ CD11b⁻ CD4⁻ TCRβ⁻ FoxP3⁻ RORγt⁺) and Th17 (CD45⁺ CD11b⁻ CD4⁺ TCRβ⁺ FoxP3⁻ RORγt⁺) cells in the lamina propria of ileum and colon. (C) proportion and absolute number of 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 $CD4^-$ $TCR\beta^-$ live cells for ILC3 and on $CD4^+$ $TCR\beta^+$ live cells for Th17 cells. (D) Same as (C) but for colonic lamina propria, including the percentage of Treg cells. N= 7-8 mice per group, representative of 2 independent experiments. *P*-values calculated by t-test *<0.05, **< 0.01. ***< 0.001, ****< 0.0001.

4.8 Effect of cross fostering on LOS susceptibility and immune phenotype

As it was, our experimental model did not allow us to separately understand the effect of i*n utero* events from that of the postnatal period and of breastfeeding, in the context of prenatal Abx therapy. Indeed, it is known that bacterial components and metabolites can cross the placenta **(Ganal-Vonarburg et al., 2020)**, while the passage of whole live organisms is still a matter of debate. Thus, it is conceivable that Abx administered to pregnant mothers may have a significant effect on the development of the fetal immune system starting before birth, and not only through the influence of breastfeeding. To gain insight into how prenatal and postnatal events could determine the outcomes we observed in the offspring, we established an experimental model of cross-fostering pups as soon as possible after birth, so that untreated ("Ctrl") mothers would have breastfed pups exposed *in utero* to Abx ("Ctrl-Abx" pups) and Abx-exposed, dysbiotic dams ("Abx") would have breastfed pups never exposed to Abx *in utero* ("Abx-Ctrl" pups) (**Figure 16A)**. For what concerns the amount of fecal IgA, in this experimental setting at P7 the key factor influencing the amount of GI sIgA was clearly breastfeeding (**Figure 16B**): 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 to the control group. After 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 to pups exposed to Abx *in utero* but breastfed by control mothers. This data was mirrored by the quantification of IgA in the stomach content of the same pups, despite the degree of separation between the groups was not of the same magnitude of fecal IgA. We then challenged pups treated with the same experimental protocol and cross fostered at birth with *E.coli K1* (**Figure 16C**). This was a single experiment, not yet replicated: we could identify a trend towards improved survival in pups fed by control mothers (green lines), i.e. with an IgA-rich milk, even if they were born to an Abx-treated (green dashed line), compared to those fed by an Abx-treated mother (yellow lines), either born to a control (yellow dashed) or Abx treated (yellow solid line). The experiment will be repeated to increase the samples size. For what concerns long term outcomes, i.e. outcomes at 28 days of life, we focused again on the most significant data obtained, i.e. IgA production by lamina propria plasma cells of the ileum. At P28, we confirmed a reduced amount of fecal IgA in young adult mice born to and raised by Abx-treated dams compared to untreated ones (**Figure 16D**, left half of the graph), and a corresponding reduction in IgA^+ plasma cells of the ileum lamina propria (**Figure 16E**, left half of the graph). After cross fostering, mice born to Abx-treated mothers, but breast fed by untreated foster mothers presented a proportion of IgA^+ plasma cells in the lamina propria of the ileum that was similar to 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 a significantly lower proportions of IgA^+ plasma cells (**Figure 16D**, right half of the graphs). However, this trend was not completely mirrored by the absolute concentration of IgA assessed in the fecal pellets, where both cross-fostered groups of young adults presented fecal IgA levels similar to animals born to and raised by previously untreated mothers,

and higher than animals born to and raised by Abx-treated mothers (**Figure 16E**). Overall, these data highlight the importance of the breastfeeding period, 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 re-establish the normal development of lamina propria IgA-producing plasma cells in the context of prenatal Abx treatment. Conversely, the vertical transmission of normal microbiota before birth (and in the first postpartum hours, since cross-fostering cannot be immediate for technical reasons) is not sufficient *per se* to ensure a normal number of small intestine plasma cells if mice are fostered after birth by a previously Abx-treated mother. Despite lower in number, these cells seemed anyway capable of producing fecal IgA in similar amounts to the other experimental groups. In conclusion, with preliminary data, we confirmed that the increased susceptibility to LOS of pups born to an Abx-treated mother depend on low levels of breastmilk IgA, and that prenatal Abx treatment seems to have long lasting effects on the small intestine IgA-producing plasma cells of the offspring, with a predominant role for breastfeeding and postnatal microbiome transmission compared to the vertical maternal-neonatal transmission of microbiota during the delivery.

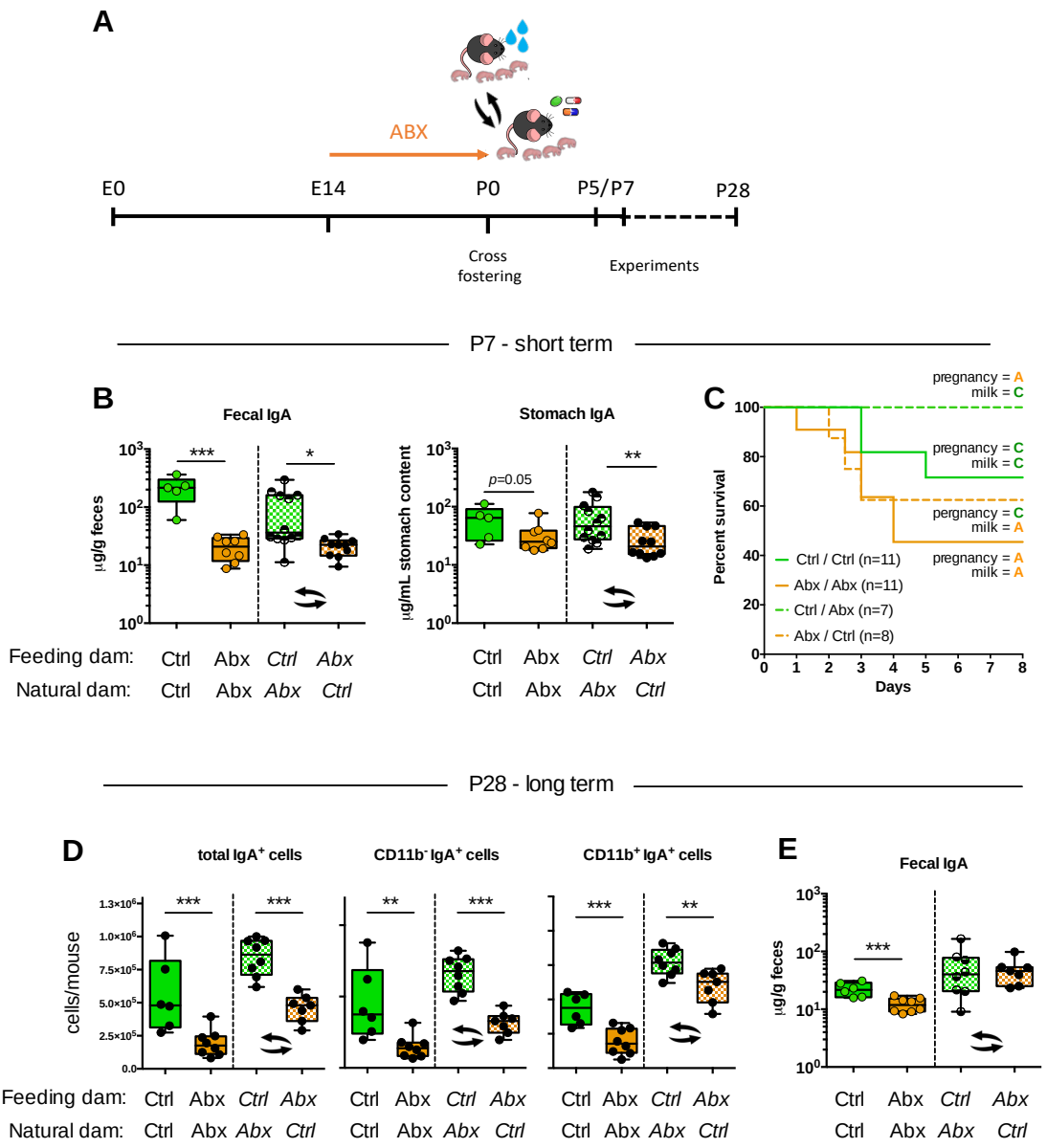

Figure 16. (A) Timed-pregnant dams were treated with Abx or left untreated from E13.5 to E18.5. Within 12 hours of birth, litters were cross fostered, and experiments were conducted at P5-7 or P28. (B) Amount of fecal IgA and stomach IgA in pups of 4 different experimental groups. N= 7-8 mice per group, representative of at least 2 independent experiments. *P*-values calculated by two-way ANOVA with Sidak correction for multiple comparisons. *<0.05, **< 0.01. ***< 0.001. (C) Survival curves of pups of the 4 experimental groups after gavage at P4-5 of 1.6 * 10^9 CFUs of *E.coli K1* (D) 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. ***< 0.001. (E) 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. ***< 0.001.

4.9 Modification of intestinal microbiota induced by prenatal antibiotics

Considered the tight, bidirectional relationship existing between lumina IgA and the gut microbiota, we reasoned that our analysis of the impact of prenatal antibiotics on neonatal GI IgA should have been completed by the analysis of fecal microbiota in our experimental groups. We selected for the analysis two timepoints after birth, P7 (when pups are exclusively breast fed) and P28, when weanlings are exclusively on a solid food diet. We investigated the bacterial composition of frozen fecal pellets through 16S rRNA gene sequencing, analyzing both α-diversity, β-diversity, and the bacterial genera composing the intestinal microbiota. Results are reported in **Figure 17**. At P7, differences in α-diversity metrics (Shannon and Faith indexes) between experimental groups were moderate, with a significantly lower Faith index in pups born and raised by Abx-treated mothers compared to the control group. The Bray Curtis cluster analysis of compositional differences between experimental groups 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, and close to pups born to a control mother but raised by Abx-treated mothers (purple dots). 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 (**Figure 17D**) confirmed that pups born to or raised by Abx mother 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. At P28, the differences between experimental groups and the influence of breastfeeding mothers became more striking. Regardless of the natural mother, pups raised (and breastfed) by untreated mothers presented a significantly higher microbial diversity compared to pups fed by Abx-treated mothers (**Figure 17E-G**), and the treatment of the feeding mother determined the 40.58% of microbial diversity at Bray Curtis cluster analysis. Relative abundance of bacterial genera in fecal pellets 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 (**Figure 17H**). In particular, being fed by untreated mothers favored a higher complexity of 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 (this last predominantly in pups cross fostered and raised by an Abx-treated mother) genera.

Overall, the trends identified by 16s rRNA sequencing of intestinal microbiota confirmed the absolute importance of breastfeeding in shaping the content of GI lumen, especially in case of prenatal administration of antibiotics.

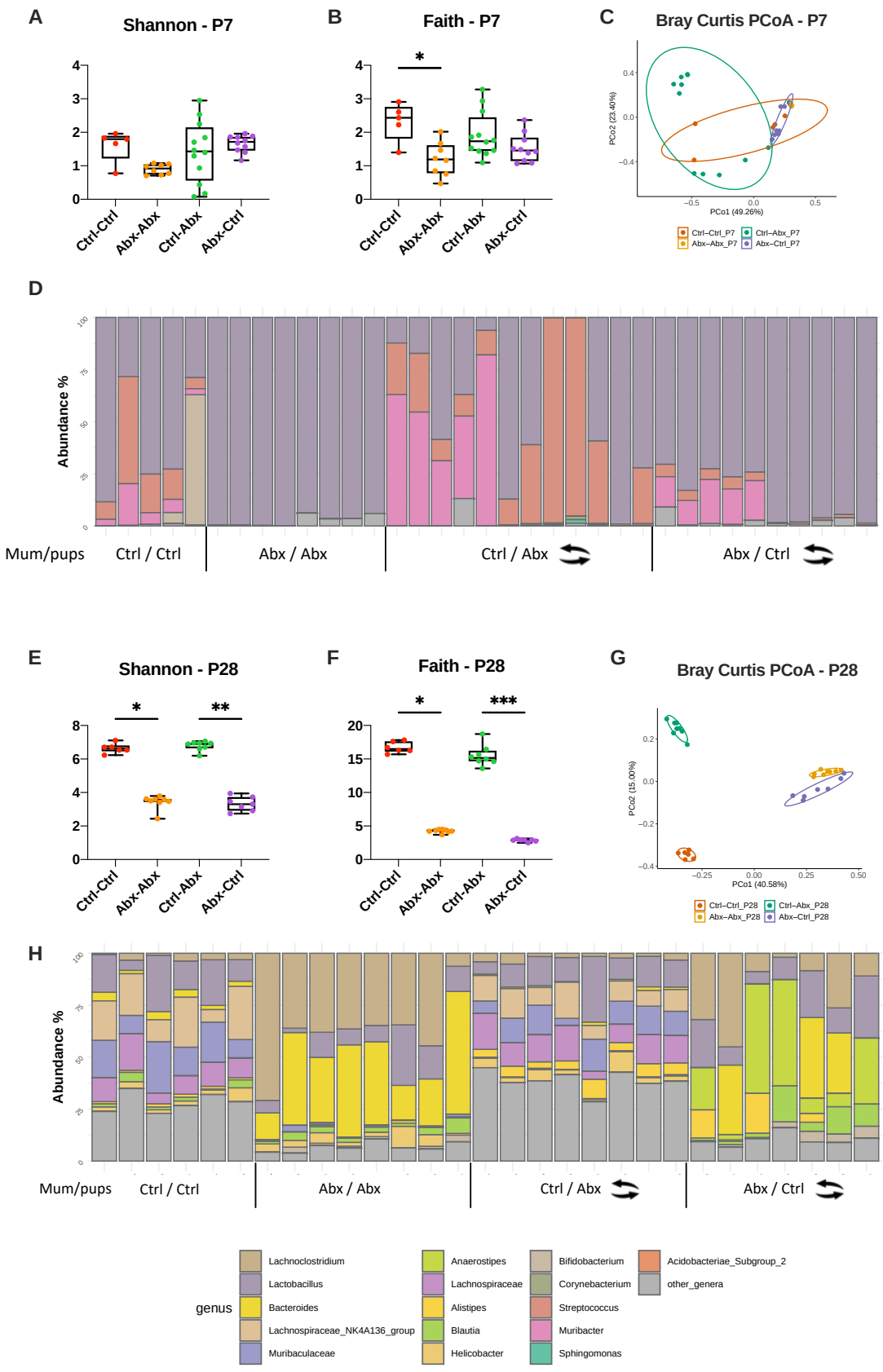

Figure 17. (A,B,C) Alpha-diversity (Shannon index and Faith index) and Beta-diversity (Bray Curtis principal component analysis) metrics of fecal microbiota at P7 in the 4 experimental

group (indicated as mother/pups). (D) Barplots depicting the bacterial general of fecal pellets in the 4 experimental group (indicated as mother/pups). (E,F,G) Alpha-diversity (Shannon index and Faith index) and Beta-diversity (Bray Curtis principal component analysis) metrics of fecal microbiota at P28 in the 4 experimental group (indicated as mother/pups). (H) Barplots depicting the bacterial general of fecal pellets in the 4 experimental group (indicated as mother/pups). N= 5-12 mice per group, representative of 2 independent experiments. *P*-values calculated by Kruskall-Wallis test with Dunn's correction for multiple comparisons. *<0.05, **< 0.01, ***< 0.001

4.10 Effect of maternal supplementation with postbiotics on the immune phenotype induced by prenatal antibiotics

Considered how frequently Abx are prescribed during pregnancy, any intervention that may improve the Abx-induced immune phenotype in the offspring should deserve attention. Here, we investigated whether a lyophilized postbiotic named immunoFOS® (iFOS), could improve the immune phenotype in the offspring when co-administered to pregnant mothers receiving Abx therapy, in a supplemented diet. To control for unspecific effect of the chemicals, specifically maltodextrins (MDX), that are added to iFOS during the lyophilization process, we also administered to a separate group of pregnant animals treated with prenatal Abx an MDX-supplemented diet. All the treatments, i.e. Abx, iFOS, and MDX, were administered between E13.5 and E18.5, and we started our investigation from the long-term outcomes, i.e. the analysis of the effect at day of life 28. Further short-term experiments, including the sepsis challenge, are yet to be completed. As reported in **Figure 18A-D**, prenatal Abx again significantly reduced the total amount of fecal IgA at 28 days of life, as well as the presence of IgA^+ plasma cells, both $CD11b^+$ and $CD11b^-$, in the lamina propria of the small intestine. This phenotype was supported by both confocal microscopy

and multiparameter flow cytometry analysis. Unfortunately, the effect of iFOS supplementation was non significant on the immune parameters so far investigated: in terms of total IgA (**Figure 18A**), iFOS induced only a mild increase compared to the supplementation with MDX, but was not sufficient to revert the phenotype induced by prenatal Abx. Furthermore, this mild increase was not mirrored by a corresponding increase in the amount of IgA^+ cells in the small intestinal lamina propria, nor at the confocal microscopy analysis, neither at the FACS (**Figure 18B-D**). Then, we investigated by means of FACS the effect of iFOS supplementation during prenatal Abx treatment on the amount of Th17 $CD4^+$ T-cells and ILC3, in the lamina propria of the small intestine and of the colon. The results replicated the differences between young adults born to Abx-treated or untreated mothers shown in **Figure 15C-D**, but confirmed the lack of significant effect of maternal iFOS supplementation for all the parameters analyzed. As a representative, we report in **Figure 18E-F** the dot plots and the quantification, both absolute and as percentage of $CD4^+$ T cells, of Th17 cells in the small intestinal lamina propria. Thus, at least for what concerns the long-term effects, we concluded that a maternal supplementation with iFOS during prenatal Abx therapy was not sufficient, in our experimental setting, to revert the pathological phenotype induced by prenatal Abx.

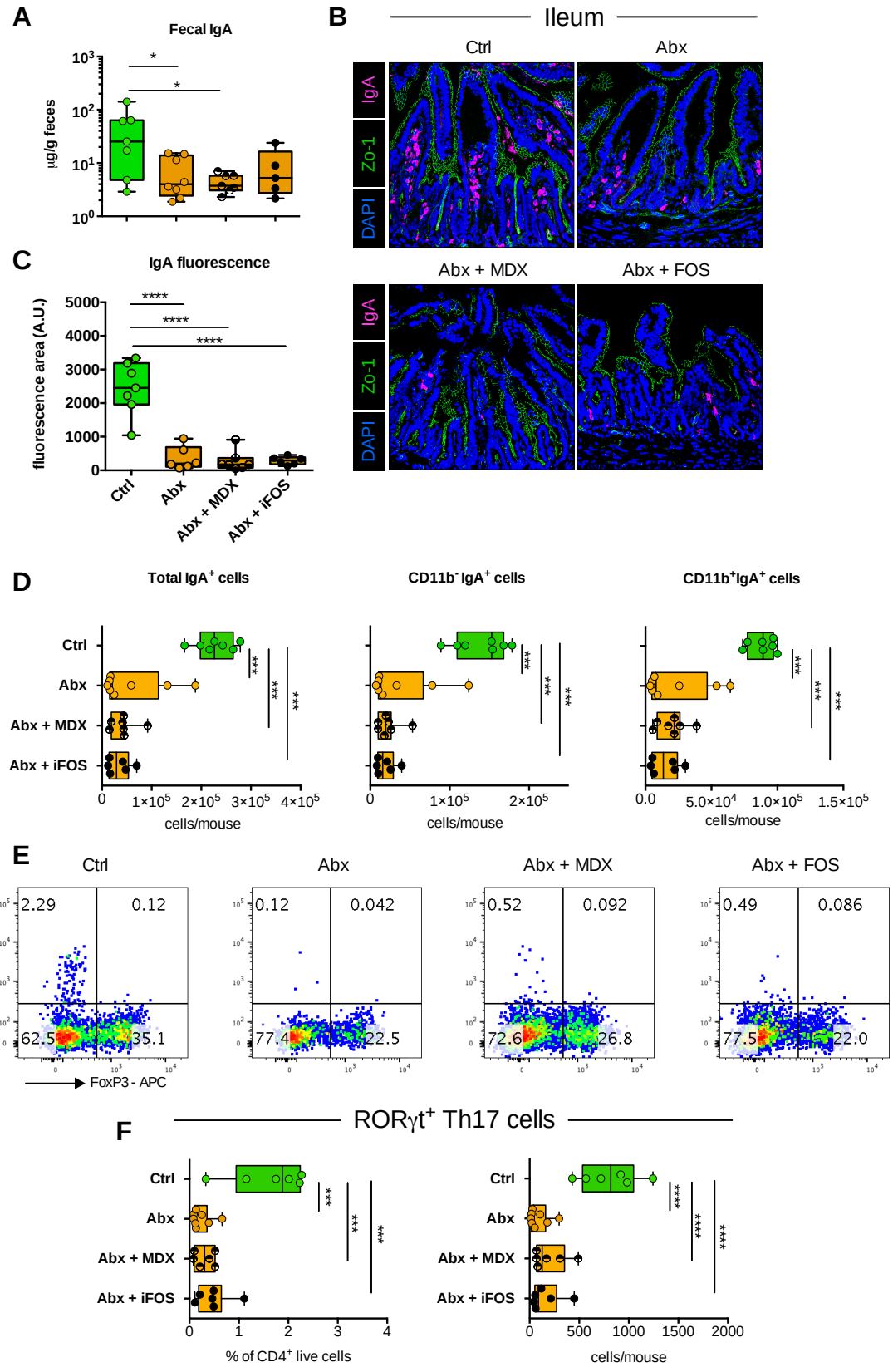

Figure 18. (A) Amount of fecal IgA at P28 in the offspring of untreated mothers (Ctrl) or of mothers treated with Abxs (Abx), Abxs + maltodextrins (MDX) or Abxs + immunoFOS (iFOS)

from E13.5 to E.18.5. (B) Representative confocal microscopy images of ileal mucosa at P28 in the 4 experimental groups. (C) Quantification of IgA fluorescence of (B). (D) Amount of total $CD45^+$ IgA^+ cells, of $CD45^+$ IgA^+ $CD11b^+$ and $CD11b^-$ cells of in the small intestine lamina propria of mice in the 4 experimental groups at P28. (E) Representative flow cytometry dot plots of Th17 $CD4^+$ T-cells in the small intestine lamina propria of mice in the 4 experimental groups at P28. (F) Percentage (of Th cells) and absolute number of Th17 cells in the small intestine lamina propria of mice in the 4 experimental groups at P28. For (A, C, D, F): N= 6-7 mice per group, representative of 2 independent experiments. *P*-values calculated by two-way ANOVA with Sidak correction for multiple comparisons. *<0.05, ***< 0.001, ****< 0.0001

Chapter 5: Discussion

Antibiotics remain among the medications most frequently prescribed during pregnancy, and it has been calculated that up to one third of pregnant women receives at least one antibiotic prescription during pregnancy **(Bookstaver et al., 2015; de Jonge et al., 2014; Santos et al., 2010)**. Indeed, at least epidemiologically, the exposure to antimicrobials during fetal life has a significant impact on the offspring, but their biological consequences have been only marginally investigated. Furthermore, in most cases antibiotics are prescribed during the last stages of pregnancy, for reasons such as the prophylaxis of GBS colonization, the treatment of an infection-triggered preterm labor, or the prophylaxis of PROM. This timing has two main consequences: first, pregnant mothers are more frequently exposed to antibiotics during the stage of highest permeability of the placental barrier and, at the same time, an important milestone for the development of the fetal immune system. After 32 weeks of gestational age, indeed, neutrophils appear in the circulation of human fetuses, as well as myeloid derived suppressor cells (MDSCs), while T-reg lymphocytes diminish to prepare the encounter with commensal microbes **(Zhang et al., 2017)**: thus, the effect of maternal antibiotics on microbial components and possibly live microorganisms that cross the placenta at this time can impact the fetal immune system in a key window of development.

Second, as they are administered close to the delivery, the effect of prenatal antibiotics can extend into early postnatal life, as antibiotic-induced dysbiosis is not immediately resolved with the interruption of antibiotic administration. Here, we discovered that a cocktail of ampicillin, neomycin and erythromycin administered to female mice during the last week of pregnancy induced a marked state of dysbiosis in the mother and in the pups, reduced the transmission of sIgA through the entero-mammary pathway to the suckling newborn, increased the susceptibility to LOS caused by *E.coli* during the first

week of life, and significantly impaired the maturation of neonatal intestinal immune system, with effects persisting beyond period. It's important to note that our experimental setting differed from most published models of perinatal dysbiosis **(Deshmukh et al., 2014; Leclercq et al., 2017; Miyoshi et al., 2017; Singer et al., 2019)**, as we exposed pregnant mothers to a short course of antimicrobial treatment that was interrupted at E18.5, with no direct exposure of newborns to antibiotics after birth through breastmilk.

A qualitative analysis of the maternal dysbiosis induced by antibiotic therapy was not an aim of our investigation, nonetheless we found a quantitative reduction of both aerobic and anaerobic bacterial species in the fecal pellet of antibiotic-treated mothers, a reduced synthesis of antimicrobial proteins and peptides in the intestinal wall, a significant increase in the mucus production, but no effect on the epithelial structure and TJs. These results confirm a robust body of previously published literature **(Brandl et al., 2008; Wlodarska et al., 2011)**, and despite some authors have previously reported a disruption of TJs after antimicrobial treatment **(Feng et al., 2019)**, these alterations could be explained by a more prolonged exposure to broader-spectrum combinations of antibiotics. Overall, the maternal phenotype we report highlights how even a short course (5 days) of clinically relevant antibiotics can have a substantial impact on maternal gut microbiome and intestinal physiology. Furthermore, the effect of maternal intestinal dysbiosis was extended to the mammary gland, where we revealed a previously unreported reduction in IgA^+ cells and sIgA excretion in breastmilk. It is known that IgA^+ plasma cells of the mammary gland originate from the MLNs, and migrate through the so called "entero-mammary pathway" driven by the chemokine gradient of CCL28 expressed by the epithelial mammary tissue itself **(Roux et al., 1977; Wilson and Butcher, 2004)**. As we mainly focused on the offspring, we haven't analyzed the pool of intestinal and MLNs IgA^+ plasma cells in antibiotic

treated or untreated mothers yet, nor the migration dynamics through the entero-mammary pathway. It will be the aim of future experiments. Nevertheless, and even more in light of the long-term effect of prenatal antibiotics on IgA^+ plasma cells of the offspring that we discovered, it seems plausible that the maternal intestinal dysbiosis causes a down regulation of LP and MLNs IgA-producing PCs in the pregnant mother, and a subsequent reduced migration towards the mammary gland during lactation. In addition to this, it is known that the mammary gland harbors its own microbiota **(Fernandez et al., 2020)**. As the antimicrobials we used are all adsorbed and can have a systemic effect, we cannot exclude a direct contribution of the mammary gland dysbiosis to the impaired sIgA production that we observed. Our main focus was to investigate the effect of prenatal dysbiosis on the development of systemic and intestinal immune function.

In neonatal blood and spleen, we did not find significant quantitative alterations of major immune populations, neither lymphoid nor myeloid, after maternal antibiotic treatment. Previously, it has been shown on a murine model that perinatal antibiotics significantly decrease the amount of circulating neutrophils (identified as $CD11b^+$ $Ly6G^+$) in newborn mice, through a ILC3 – G-CSF – mediated mechanism in the mucosa of the SI **(Deshmukh et al., 2014)**. However, these data have been obtained with a prolonged administration, both before and after birth, of antibiotic cocktails that included vancomycin. Furthermore, no confirmation of neonatal neutropenia after maternal antibiotic treatment mothers exists yet in humans. We speculate that the difference between previous and our results can be explained by the different experimental settings, and in particular by the more prolonged, direct antibiotic exposure of neonatal mice used in previous studies.

In our experimental model, the structure of intestinal epithelium, the expression of TJs proteins and the intestinal permeability to small molecules was also not altered in newborn mice after the exposure to prenatal antibiotics. These data closely resemble those obtained by Leclerq and coll. with the use of penicillin on a murine model **(Leclercq et al., 2017)**, and reveal that antibiotics administered only in the prenatal period, not directly to young mice, are not sufficient to induce structural alterations of the intestinal epithelium.

Despite the apparent lack of effect of prenatal antibiotics on these important aspects of neonatal immune system, we observed a significantly increased susceptibility of pups born to antibiotic-treated mice to a model of LOS induced by *Escherichia coli*, a common Gram-negative neonatal pathogen. The increased susceptibility correlated with an increased bacterial translocation to distal organs, a reduced amount of fecal IgA, of stomach IgA (a substitute for breast milk), and of bacterial coating by IgA themselves, including the specific coating of the pathogenic *E. coli*. Secretory IgA in the gastro enteric tube of newborns are largely carried by breast milk **(Lindner et al., 2012; Zhang et al., 2017)**, and represent a key protective factor against bacterial translocation **(Dickinson et al., 1998)**, neonatal sepsis **(Maxson et al., 1995)** and, as recently demonstrated, necrotizing enterocolitis **(Gopalakrishna et al., 2019)**. This protection can be conferred also in the absence of antigen-specific IgA induced by a previous exposure to or immunization against pathogens of the mother, i.e. by the broadly reacting, low-affinity IgA induced by the species composing the maternal microbiota, the so called "natural IgA" **(Pabst and Slack, 2020; Zheng et al., 2020)**. As our female mice 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 antibiotic treatment has never

been reported previously, neither in humans nor in animal models: as for any result obtained on well controlled animal model, the validation on an appropriate cohort of human neonates will be necessary to corroborate our data. Previously, Reed and coll. reported a slight increase in LOS risk (OR 1.59; 95% CI 0.84-2.99; P = .15) in a cohort of very preterm neonates exposed to prenatal antibiotics **(Reed et al., 2018)**. Nonetheless, the authors themselves pointed out how difficult it can be to clarify the effect of prenatal antibiotics in a real-life scenario, where mothers are exposed to different molecules over a different time period, and where neonates born to antibiotic-treated mothers are frequently treated themselves with antibiotics. We are aware that the correlation we report between maternal antibiotic treatment, low breastmilk/fecal IgA and increased *E. coli* translocation/susceptibility to LOS did not represent a proof of causality. Thus, to gain insight into the causative role of low breast milk IgA, we performed cross-fostering experiments. The preliminary results supported our hypothesis. Indeed, the levels of fecal and stomach IgA in the first days of life were reduced in pups breastfed by an antibiotic-treated mother, and these pups were more susceptible to LOS compared to those breastfed by an antibiotic-untreated mother. This was a single experiment, to be repeated, and it should also be considered that other factors altered by the antibiotic treatment may contribute to the susceptibility to LOS. In particular, as the intestinal niche is a complex ecosystem **(Rao et al., 2021)**, it is more than likely that maternal antibiotics can directly alter the colonization dynamics of neonatal gut, both before and after birth, as well as the relationship and the competition between the assembling intestinal microbiota and the *E.coli* used to model LOS. 16S rRNA gene sequencing analysis of fecal microbiota in the different experimental groups is ongoing, and will help to unravel the possible contribution of single bacterial species altered by maternal antibiotics to the functional phenotype we report.

Furthermore, the cross-fostering strategy is not a perfect model to isolate the role of IgA from that of dysbiosis, as we could not separate the pups from their natural dam immediately after birth. Therefore, vertical transmission of maternal microbiota during delivery and a short period of breast feeding from the natural mother could not be completely avoided, making the cross-fostering strategy suboptimal. The use of transgenic mice knockout for IgA **(Mbawuike et al., 1999)**, or of germ-free mice could both be useful to understand the pure role of breast milk sIgA in the context of prenatal antibiotic therapy, and independently from microbiome variations, but these strategies are currently beyond our possibilities.

In a second set of experiments, we analyzed the effect of prenatal antibiotic at a longer time point, 4 weeks of age, to ascertain whether prenatal antibiotics could also impact the progressive maturation of the neonatal intestinal immune function and have longer lasting effects. Indeed, we reasoned that the assembling gut microbiota, breast milk sIgA and the mucosal intestinal immune system "in training" during early life formed a three-way, mutual connection whose players are tightly tied **(Gensollen et al., 2016; Torow et al., 2017)**. When a perturbation alters one of these three components, it is reasonable to expect modifications in the others. 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, and to shape several details of its functionality, such as the antigen-specificity of plasma cells, the balance between T-helper populations in the mucosa, and the development of innate ILCs, in particular ILC3 ($ROR\gamma t^{+}$ Lin^{-} $NKp46^{+}$) **(Guo et al., 2021; Kalbermatter et al., 2021)**.

In young adult mice born to and raised by antibiotic-treated mothers we found a previously unreported reduction in fecal sIgA, as well as a reduced

absolute number of both $CD11b^+$ and $CD11b^-$ IgA^+ plasma cells. It has been recently shown that intestinal IgA^+ $CD11b^+$ plasma cells, compared to 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 **(Kunisawa et al., 2013)**. 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 in early 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 antibiotic-treated mothers, just before the $CD11b^-$ fraction of plasma cells became predominant in the LP. The cross-fostering experiments confirmed the predominant role of the fostering mother, and thus of breastmilk IgA (and possibly microbiota), in shaping the development of intestinal immune cells and of the intestinal microbiota. Overall, the breastfeeding window is key to maintain the homeostasis of gut immune system development later in life. To gain further insight into this phenomenon, we are planning future experiments to analyze the RNA transcription by the cells of the SI mucosa of cytokines known to promote the development of plasma cells.

In our model, other cellular population of the SI LP were affected by prenatal antibiotic treatment. First, we identified a reduction in LP dendritic cells (DCs, $MHCII^+CD11b^+CD11c^+CD103^+$) and mononuclear phagocytes (MNPs, $MHCII^+CD11b^+CD11c^-CD103^-CXCR1^+$) at 28 days of life after prenatal antimicrobial treatment. 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-cells stimulation (DCs), and stimulate LP

plasma cells to produce sIgA, either with the assistance of Th cells or in a T cell – independent manner **(Ko and Chang, 2015)**. Furthermore, the development of both MNPs and DCs is favored by the presence of a normal intestinal microbiota, and hampered by antibiotic treatment **(Honda et al., 2020)**: we recapitulated a similar phenotype even without a direct exposure of pups to antibiotics, but with the vertical transmission of a dysbiotic phenotype and the reduction of luminal IgA. Specifically, we hypothesize that the reduced amount of LP $CD103^+$ DCs hindered the development of LP plasma cells that we report. Further experiments with knockout mice could be useful to confirm or not this hypothesis.

Second, we observed a reduction in colonic Tregs, and in both SI and LI $TCR\beta^+CD4^+FoxP3^+ROR\gamma t^+$ cells after prenatal antibiotic treatment. We defined these cells as "Th17" *bona fide*, despite a more detailed FACS panel including other transcription factors such as GATA3 and T-bet will be needed to corroborate these results. If this trend will be confirmed, it represents another long-term effect of the prenatal antibiotic therapy. Recently, it has been shown on a murine model that a prenatal, transient infection of a pregnant mother increases the amount of Th17 cells (defined as $TCR\beta^+CD4^+FoxP3^+ROR\gamma t^+T\text{-}bet^-$) in the SI LP of the offspring in adult age, through epigenetic changes on fetal intestinal epithelial stem cells imposed by maternal IL-6 **(Lim et al., 2021)**. LP Th17 cells produce IL-17 and IL-22, and at the mucosal interfaces they contribute to inflammatory responses against pathogens **(Omenetti and Pizarro, 2015)**. In light of this evidence, we speculate that the model of prenatal antibiotic therapy functionally represents a reflection of the effect reported by Lim and coll., with possible long-term consequences (e.g. an increased susceptibility to infections by enteric pathogens) that will be the focus of future experiments. Furthermore, the 16S rRNA gene sequencing analysis of fecal microbiota and the transcriptional analysis of cytokines

produced by the intestinal mucosa, which are both ongoing, will help to clarify the role of specific microbial species and cytokines in the reduction of the Th17-like population.

Finally, in the last part of our project we aimed to investigate possible therapeutic options to limit the dysbiosis-induced phenotype in the offspring of antibiotic-treated mothers. Indeed, prenatal antibiotics are a critical weapon to fight infections and to prevent fetal/neonatal colonization by pathogenic bacteria during pregnancy. Even though a judicious administration is more than desirable, they are frequently unavoidable. Thus, a possible therapeutic approach could include the use of nutritional supplementations able to counteract the effect of antimicrobials. We focused our attention on iFOS®, an industrial dried soluble product of *Lactobacillus paracasei CNCM I-5220* aerobic fermentation that has previously shown significant immunomodulatory effect *in vitro* and *in vivo* **(Zagato et al., 2014)**. In our first set of experiments, we administered iFOS® to pregnant mothers simultaneously with antibiotic therapy, to mimic a possible therapeutic approach in human women. Unfortunately, with this experimental setting, we could not observe any significant improvement of the antibiotic-induced phenotype at 28 days of life, neither in terms of fecal IgA, nor of IgA^+ plasma cells of the intestinal LP, nor of Th17-like cells. We reasoned that, in general, the biological effect of iFOS® might have been too mild compared to the striking abnormalities induced by the antibiotic cocktail. Alternatively, as *Lactobacillus paracasei* metabolic products are known to have also a pre-biotic effect, mediated by the intestinal microbiota **(Roggero et al., 2020)**, it is conceivable that the immuno-stimulatory effect of iFOS® would be more relevant in the context of microbiota reconstitution, after the interruption of antibiotic therapy. Further experiments with iFOS® administration to dams

during pregnancy and breastfeeding, i.e. during the phase of microbiota reconstitution, will better clarify the possible immuno-modulatory effect of iFOS® in the context of prenatal antibiotic therapy.

In conclusion, we here propose that prenatal antibiotics administered to pregnant mothers during the last stage of pregnancy profoundly increase the susceptibility of neonates to LOS, through the reduction of breastmilk sIgA mediated by the dysbiotic environment in the mother. The effects of prenatal antibiotics on the offspring persist into young adulthood, where the production of sIgA by SI LP plasma cells is reduced after maternal antibiotic therapy, as well as the number of Th17 mucosal lymphocytes. Additional investigations are required to further dissect the relationship between sIgA and microbiota, as synergistic factors that can affect the development of neonatal intestinal immune system in the context of prenatal antibiotic therapy.

The clinical implications of our data may be relevant: 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 antibiotics 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 antibiotic therapy before delivery might prove beneficial to select the most appropriate bank milk for each preterm neonate who needs for it, or to evaluate a possible supplementation of breast milk low in sIgA, with the ultimate goal to reduce the incidence of harmful events such as LOS and NEC in extremely preterm neonates.

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