Gut maturation and early life antibiotics

An impact for life?

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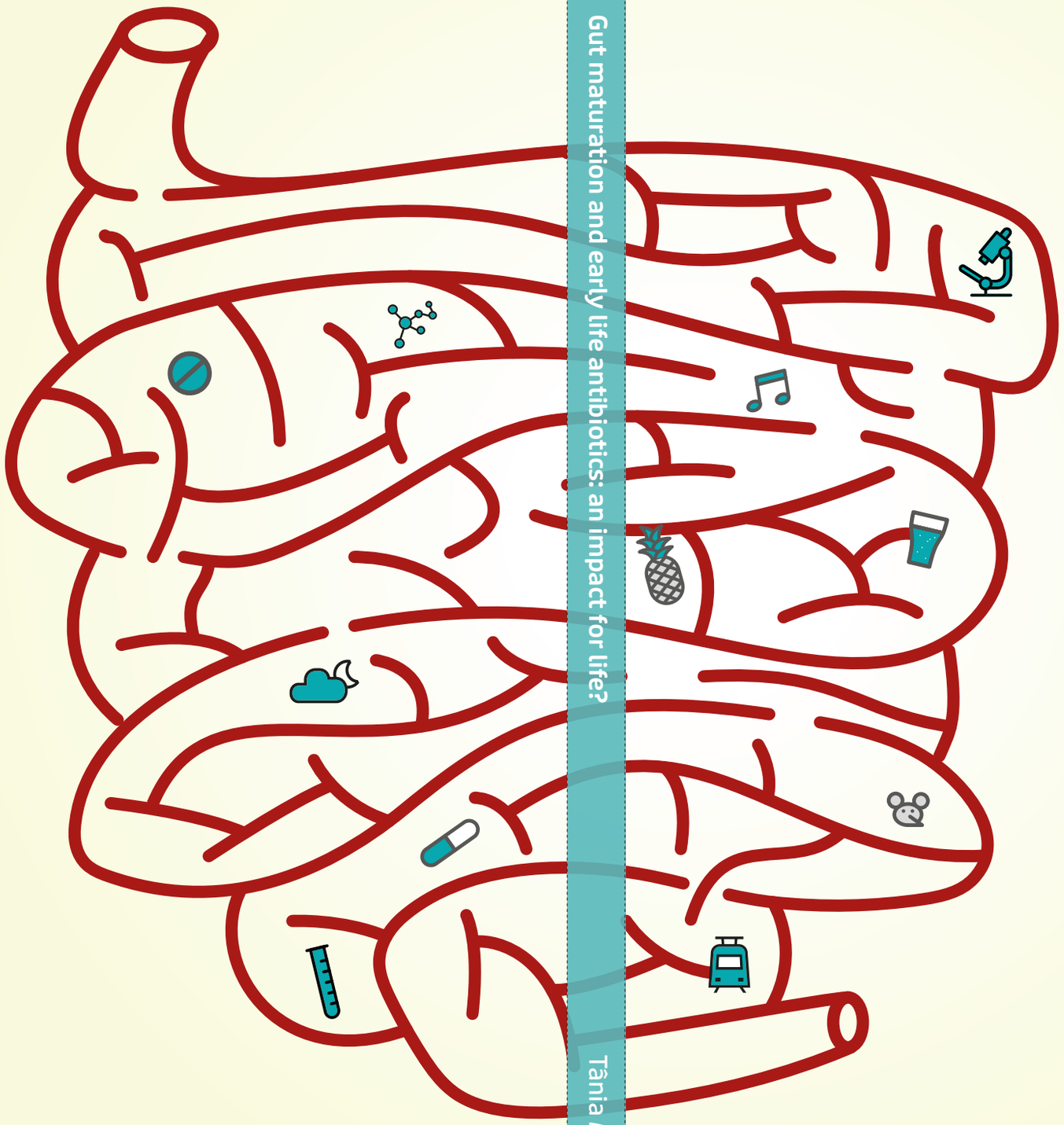
Publication date
2022

Document Version
Final published version



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Layout and printed by: Optima Grafische Communicatie (www.ogc.nl)
ISBN: 978-94-6361-709-3

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ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. ir. K.I.J. Maex
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Aula der Universiteit
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Faculteit der Geneeskunde

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General Introduction

THE SIMPLE COMPLEXITY OF THE SMALL INTESTINE

The small intestine (SI) is a tubular structure extending from the stomach to the colon, positioned within the abdominal cavity. The four layers composing the SI, from the outer part to the lumen side, are serosa, muscularis, submucosa, and mucosa. The serosa protects the inner layers, while the muscularis layer is responsible for intestinal motility. The submucosa binds the muscularis to the mucosa and consists of connective tissue filled with blood and lymphatic vessels and nerves. The mucosa is the innermost layer, which is in contact with the lumen, and is constituted by the epithelium, the lamina propria, and the muscularis mucosae (Figure 1). The intestinal epithelium is a single layer of polarized epithelial cells responsible for the absorption of food nutrients necessary for the functioning of all cells of the organism. At the same time, the SI needs to prevent the translocation of pathogens from the lumen to the blood, which is achieved by the presence of a selective barrier that keeps absorption and defense in balance. This barrier consists of a luminal mucus layer and antimicrobial peptides secreted by epithelial cells, the epithelium, and immune cells. In addition, commensal bacteria present in the lumen and in the mucus layer form the first layer of defense as they prevent the growth of pathogenic microorganisms.

The SI epithelium has a distinct morphology. It extends deep into the mesenchyme, forming crypts, and, at the same time, protrudes to the lumen, giving rise to finger-like structures named villi (Figure 1). While the crypt compartment constitutes the morphological niche for the proliferative cells, the villus compartment is composed of differentiated cells. The pluripotent stem cells located at the base of the crypt proliferate and continuously supply new daughter cells that differentiate while migrating to the top of the villus in a timeframe of 5 to 7 days after which they are extruded [1-4]. This makes the small intestine the organ in our body with the fastest cell turnover, which serves as an important protective mechanism from the high risk of cellular damage caused by infectious organisms or harmful compounds [5].

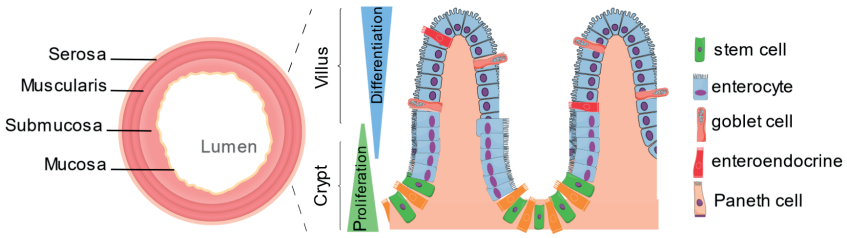

Figure 1 – Schematic representation of the small intestine and detail of its epithelial layer.
Four layers form the walls of this tubular structure. The epithelium is part of the innermost layer and it is formed by an endless wave of proliferative crypts and differentiated villi. The main cell types composing the epithelium are the absorptive enterocytes, the secretory goblet, Paneth, and enteroendocrine cells and lastly the stem cells.

There are four major differentiated cell types in the SI (Figure 1). Around 80% of the intestinal epithelial cells (IECs) are absorptive enterocytes. Enterocytes take up and further hydrolyze the small molecules that result from the extensive breakdown of dietary carbohydrates, proteins, and fats by enzymes present in the oral cavity and stomach. The apical side of the enterocytes is fully packed with microvilli, which resemble a fringe on the intestinal lining. They increase the surface area, making the absorption of small molecules more efficient. The enterocytes microvilli constitute the intestinal brush-border. Here, different enzymes are responsible for the hydrolysis of oligosaccharides into glucose, fructose, and galactose, which are then absorbed by various transporters [6]. Furthermore, peptidases located at the brush-border and in the cytoplasm of the enterocytes hydrolyze dipeptides and tripeptides into amino acids [7]. Moreover, fatty acids and monoacylglycerols enter the enterocytes, helped by mixed micelles and specific transporters, and enzymes at the cytoplasmatic surface of the endoplasmic reticulum re-synthesize them into triacylglycerols [8]. Most of the nutrients are then exported to the blood circulation.

The other main IEC types forming the SI epithelium belong to the secretory family: goblet cells, enteroendocrine cells, and Paneth cells. Goblet cells synthesize and secrete components, such as the mucin Muc2, that constitute the mucus layer on the luminal side of the epithelium, essential to prevent or limit the contact between bacteria and IECs [9]. Enteroendocrine cells secrete several hormones, at varying levels, in response to food products and bacterial stimuli. These hormones regulate food intake, digestion, insulin secretion, intestinal motility, blood glucose levels, epithelial barrier integrity, and mucosal immunity. Finally, Paneth cells are the only differentiated cell type that differentiates while migrating to the bottom of the crypt, instead of moving up the villi. They secrete antimicrobial peptides, as for example Lysozyme-1 and Reg3 lectins [10], and growth factors essential for the functioning of the stem cells, like Wnt and EGF [11].

Along the proximal-to-distal axis, three SI regions can be distinguished according to their specific functions. Typically, the proximal part of the SI, or duodenum, is responsible for iron and calcium uptake. In the middle SI, or jejunum, the majority of carbohydrate, protein and fat absorption takes place. In the distal region of the SI, or ileum, bile acids and vitamin B12 are taken up [12, 13]. The different epithelial cell types display unique properties that are region-specific. Enterocytes, Paneth, and stem cells have distinct gene expression signatures depending on their location, and goblet cell numbers increase towards the distal SI [14, 15]. The expression of hormones by enteroendocrine cell types is different according to the cell's regional location as well. For example, gastric inhibitory polypeptide (GIP) and cholecystokinin (CKK) are mostly expressed in the duodenal

enteroendocrine cells while peptide YY (PYY) and neurotensin (NTS) expressions are higher in the ileum [16, 17].

Along the crypt-to-villus axis, there is a switch from glycolysis to oxidative phosphorylation as the main energy production pathway, due to the different metabolic requirements between proliferative and differentiated cells [18]. Both metabolic pathways use glucose as the start substrate and follow the same first steps of the glycolytic pathway, responsible for the conversion of glucose to pyruvate [19] (Figure 2). Then, pyruvate can follow separate fates, mostly dependent on oxygen availability. In aerobic conditions, cells use oxidative phosphorylation and the pyruvate is oxidized in the tricarboxylic acid (TCA) cycle and the released electrons used by the mitochondria to produce ATP. In anaerobic conditions, cells choose glycolysis that converts pyruvate to lactate generating less ATP but faster, while supplying metabolites essential to many pathways involved in cell growth and proliferation.

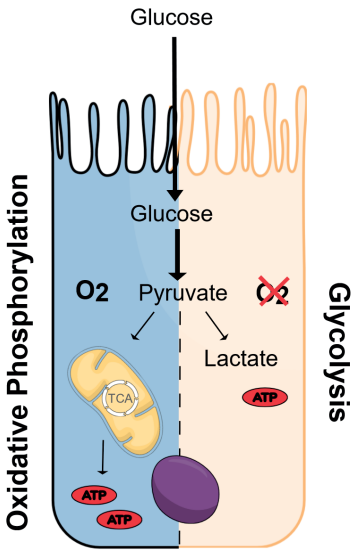

Figure 2 – Simplified overview of the two main metabolic pathways in a cell.
Glucose is used by the cell to generate ATP via two different pathways. In aerobic conditions, pyruvate is oxidized via the TCA cycle which takes place within the mitochondria. In anaerobic conditions, pyruvate is converted to lactate in the cytosol.

The complex functions carried out by the SI are only possible due to the simple but ingenious morphology of its epithelium. How the intestinal epithelium develops, from the fetal stage to adulthood, will be introduced next. As the experimental work in this thesis was performed using a mouse model, the following paragraphs will mostly focus on the mouse intestinal epithelium development.

THE THREE PHASES OF INTESTINAL EPITHELIUM DEVEVELOPMENT

During mouse embryogenesis, around embryonic day (E)9.5, the gut starts to form as a tube with highly proliferative pseudostratified epithelium originated from the endoderm [20] (Figure 3). Up until E14.5, the gut epithelium is rather morphologically similar to the stomach, displaying little to no cytodifferentiation along the proximal to distal axis. Besides, it was shown that, at this E14.5 stage, the transcriptomes of the stomach, pylorus, and intestine are only slightly different. Yet, between E14.5 and E16.5, drastic changes occur (Figure 3). First, the emergence of mesenchymal cells instructs the epithelium to self-organize to a simple columnar monolayer [21]. Second, the morphogenesis of the villi starts signaled by dense clusters of mesenchyme cells placed on the basal side of the epithelium [22]. At the start of villus morphogenesis, no distinction between the proliferative and differentiated compartments is evident as all villus cells are dividing. Later on, during development, epithelial proliferation is concentrated to the intervillus region, the morphological site of the future intestinal crypts [23]. Finally, at E16.5, hundreds of genes specific to the SI, including the widely used intestinal marker Villin, are coordinately upregulated during a process called intestinalization [24]. Among these upregulated genes are the transcription factors *Hnf4γ*, *Tcfec,* and *Creb3l3* [24]. The fetal intestinal epithelium can be distinguished by the surrounding tissue by the presence of the ubiquitous genes Cnx43 and Trop2, but their expression in the intestine rapidly decreases upon birth [25]. Other markers associated with fetal intestine have also been described, such as *Spp1*, *Ly6a*, and *Clu* [25].

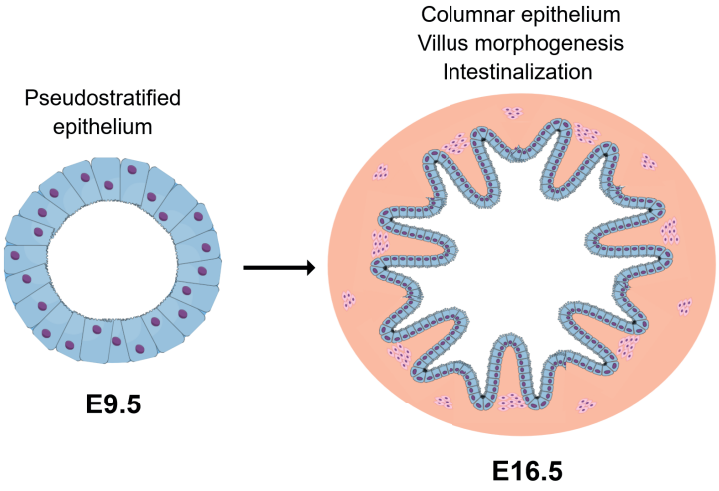

Figure 3 – The fetal stage of mouse intestinal epithelium development.
At embryonic day (E)9.5, the endoderm forms a tube organized as a pseudostratified epithelium. At E16.5, the small intestinal epithelium organizes as a simple columnar monolayer, mesenchymal cell clusters induce the formation of villi, and genes specific to the small intestine start to be expressed (intestinalization).

At birth, around E21 or E22, the mouse small intestine is still immature and birth itself imposes a drastic change in way the of nutrient supply and type of nutrition. During the prenatal phase, nutrients are mainly acquired via the umbilical cord and only limitedly through the gastrointestinal tract by absorption of nutrients from the amniotic fluid. In the neonatal phase, breastmilk provides all the necessary nutrition for the growth of the organism. Breastmilk composition changes along lactation but it is still considered a monotonous diet, mainly rich in carbohydrates, proteins and lipids [26]. Besides providing nutrients and growth factors necessary for growth, milk also contains maternal antibodies, or immunoglobulins (Ig), that constitute the first form of adaptive immunity of the neonate [27]. To guarantee the transfer of all food and immune macromolecules, the rodent immature enterocytes have tubular vacuoles on the apical side forming an apical canalicular system (ACS) [28-30] (Figure 4). In the proximal small intestine, the ACS allows the transfer of maternal Ig's from the intestinal lumen to the blood circulation, without being degraded [23, 31, 32]. In the distal small intestine, the ACS is responsible for the active endocytosis of macromolecules present in milk, which are degraded within large lysositic supranuclear vacuoles [31, 33]. The ACS and the still not fully closed tight junctions result in a higher neonatal intestinal permeability than in the adult intestine.

The neonatal intestinal epithelium is characterized by the expression of several markers (Figure 4). One of the most important markers in the neonatal stage is lactase phlorizin hydrolase (lactase). This glycohydrolase is located at the enterocyte apical side and is responsible for the degradation of lactose, the main carbohydrate source in milk. In most humans and mice, lactase expression typically increases in the late fetal developmental stage and gradually decreases after birth [5, 34-37]. Another feature of the human and mouse immature enterocytes is the expression of argininosuccinate synthase 1 (Ass1), the rate-limiting enzyme in the arginine biosynthesis [38, 39]. The semi-essential amino acid arginine is not present in milk, although it is necessary for neonatal growth. Thus, enterocytes synthesize arginine using the precursors glutamine and proline, which can be found in milk. The neonatal Fc receptor (FcRn), which mediates the uptake of IgG from breast milk, is expressed in the human SI throughout life but in mice it is only highly expressed in the neonatal intestine, sharply decreasing at weaning [40-42]. Furthermore, the expression in the mouse intestinal epithelium of cathelin-related antimicrobial peptide (CRAMP), part of the very first innate defense mechanism, is also limited to the first two postnatal weeks [43]. Finally, the expression of oxidative phosphorylation proteins in the mouse small intestine has been described to decrease during the first weeks of life [34].

At weaning, the diet becomes more varied. An intrinsic maturation process, called suckling-to-weaning transition, prepares the intestine for the gradual switch from milk

to solid food [44, 45] (Figure 4). This occurs as of 4 to 6 months of age in humans and 2 to 3 weeks after birth in mice, after which the intestine gains all the adult characteristics. In mice, the suckling-to-weaning transition has been shown to be regulated by B lymphocyte-induced maturation protein 1, or Blimp-1. This transcriptional repressor is exclusively expressed during embryonic intestinal development up until the third postnatal week and its expression is essential to keep the intestinal epithelium immature throughout these stages [46, 47]. This was demonstrated by the deletion of Blimp-1 specifically in the intestine at an early embryonic developmental stage, which resulted in accelerated epithelial maturation, including the disappearance of vacuolated enterocytes, induction of adult markers expression, and premature development of crypts and Paneth cells. The gradual loss of Blimp-1 expression during suckling-to-weaning transition allows the intestinal epithelium to mature and acquired all the characteristics of the adult intestine.

Several changes are observed in mice IECs during the suckling-to-weaning transition, occurring between P14 and P21 and following a proximal-to-distal wave [48] (Figure 4). All vacuolated enterocytes are gradually replaced by matured enterocytes without ACS [49]. The loss of vacuolated enterocytes and the closing of tight junctions leads to a more selective intestinal barrier, resulting in reduced intestinal permeability [42]. There is a decrease in the expression of proteins related to absorption and metabolism

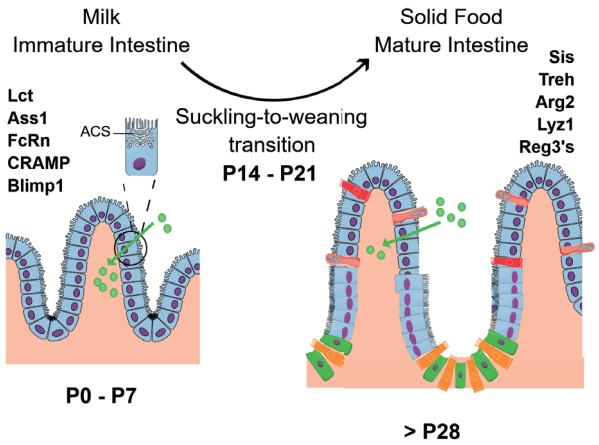

Figure 4 – The postnatal stage of mouse intestinal epithelium development.
During the first days after birth, the mouse intestine epithelium is still immature and adapted to aborption of milk components: vacuolated enterocytes due to the presence of the apical canalicular system (APC), high transepithelial permeability, expression of neonatal genes lactase (Lct), argininosuccinate synthase 1 (Ass1), neonatal Fc receptor (FcRn), cathelin-related antimicrobial peptide (CRAMP), and B lymphocyte-induced maturation protein 1 (Blimp-1). Between postnatal day (P)14 and P21, occurs the suckling-to-weaning transition characterized by the disappearance of vacuolated enterocytes, low transepithelial permeability, expression of adult genes sucrase-isomaltase (Sis), trehalase (Treh), arginase-2 (Arg2), lysozyme-1 (Lyz1), and Reg-3 lectins (Reg3's), and the development of proliferative crypts cointaining Paneth cells. By P28, the small intestinal epithelium is considered mature and adapted to digest solid food.

of fatty acids and proteins involved in oxidative phosphorylation, as well as in the gene expression of the neonatal markers lactase, Ass1, FcRn, CRAMP, and Blimp-1 [34, 41, 42, 46]. Uptake and digestion of carbohydrates increases, and monosaccharides become the main source of energy after weaning [34]. Glycohydrolases typical of the adult intestinal, such as sucrase-isomaltase (Sis) and trehalase (Treh), start to be expressed at the brush-border of enterocytes in both humans and mice [5, 44, 50, 51]. Here, complex carbohydrates are digested into monosaccharides that are then absorbed by specific transporters, as for example Slc2a1/Glut1 and Slc2a2/Glut2 [5]. Furthermore, as arginine is abundantly present in solid food, it does no longer need to be synthesized by the enterocytes. Therefore, mouse IECS begin to express arginase 2 (Arg2), the enzyme responsible for the catabolism of arginine, from birth, while Arg2 expression in the human IECs starts from birth [39, 52].

A great transformation in the mouse intestinal epithelium morphology also occurs up until weaning, with the appearance of crypts two weeks after birth (Figure 4). Crypts develop at the intervillus regions, restricting proliferation to these invaginations of the epithelial monolayer. Paneth cells finally emerge and start producing enteric antimicrobial molecules that will constitute a fundamental aspect of the intestinal innate defense: Lysozyme-1 (Lyz1) and Reg-3 lectins [43, 53-55]. The overall intestinal metabolism also changes. Expression of proteins involved in the electron transport chain that fuels oxidative phosphorylation decreases, accompanied by the rise in expression of glycolytic enzymes in enterocytes [34]. After weaning the intestine is finally considered mature.

HOW TO ADJUST THE MATURATION CODE

It was first believed that the intestinal epithelial transformations that occur during the suckling-to-weaning transition were induced by the change in diet from milk to solid food or by the establishment of commensal bacteria in the intestinal lumen [45, 56-58]. Besides, hormonal signals, both during pregnancy and early neonatal phase, were also considered as possible regulators of the suckling-to-weaning transition [59, 60]. However, several studies, mainly in the decades of the 80s and 90s, demonstrated that intestinal maturation is largely intrinsically programmed, regulated in a precise and timely manner, and largely independent of external factors [5, 61-63]. For instance, isografts of murine fetal intestine transplanted into adult mice developed normally: gave rise to crypt-villus structures, kept the correct regional patterning, and followed the same pattern of glycohydrolases expression, displaying the strongest lactase presence two weeks after transplantation and the highest sucrase presence two weeks later [61-63]. Therefore, intestinal maturation occurs in the absence of luminal or extraintestinal stimuli and

it is timely regulated by factors that are intrinsic to the intestine and specified in early development. Indeed, the above-described transcriptional repressor Blimp-1 is one of the intrinsic factors that control the moment when the intestinal epithelium starts maturing [46].

Although not necessary to induce the suckling-to-weaning transition, certain hormones and extrinsic factors present in the lumen, like microbiota and nutrition, can at least partially modulate the intestinal epithelial maturation [45, 57, 60, 64, 65] (Figure 5). One of the most studied external factors shown to accelerate intestinal maturation are glucocorticoids. Corticosterone in mice and cortisol in humans are known to increase just before birth and, in mice, again around weaning [60]. Furthermore, *in vivo* and *in vitro* treatment of neonatal mice and rats with dexamethasone, a synthetic glucocorticoid, increases the expression and activity of sucrase and trehalase in the intestine [5, 50, 59, 60, 66-69]. In humans, the glucocorticoids dexamethasone and betamethasone, amongst others, reduce the risk of NEC when given before 34 weeks of gestation [70-72]. The intestinal microbiota can also regulate certain aspects of epithelial maturation. For example, the pattern of glycosylation of epithelial-specific proteins changes from predominantly sialic acid to fucose at the terminal end [73, 74]. This is accompanied by an increase in fucosyltransferase activity, which is not observed in germ-free mice, i.e. mice that lack microbiota [75, 76]. Finally, the type of nutrition is another external factor capable of influencing intestinal development. The SI is capable of adapting to precocious weaning, by increasing the activity of adult intestinal brush border enzymes and decreasing lactase activity [77]. In addition, components present in the diet, such as growth factors, oligossacharides and hormones present in human milk, are similarly able to modulate intestine maturation [78, 79]. For example, the epidermal growth factor (EGF) is abundant in human milk and has been shown to increase villus height and crypt depth and enhance proliferation, contributing to the development of the intestine [80]. There is also evidence that insulin-like growth factor (IGF), fibroblast growth factor 21 (FGF21), and glucocorticoids can support intestinal development [81-83]. Lastly, human milk oligosaccharides (HMO) are non-digestible carbohydrates that can promote intestinal maturation by either interacting directly with IECs or after being metabolized by the commensal microbiota [84, 85].

In certain conditions, intestinal maturation can be delayed or impaired, for example in case of preterm birth, undernutrition or infections, which cause severe complications in humans [86-91]. Inappropriate intestinal maturation can lead to exacerbated inflammation of the neonatal gut and in preterm born infants ultimately resulting in the development of sepsis and necrotizing enterocolitis (NEC) [70, 92-94]. In addition, the

exacerbated inflammation can lead to stunting and impaired neurological development which not only affects health acutely, but also has long life health consequences [70, 95].

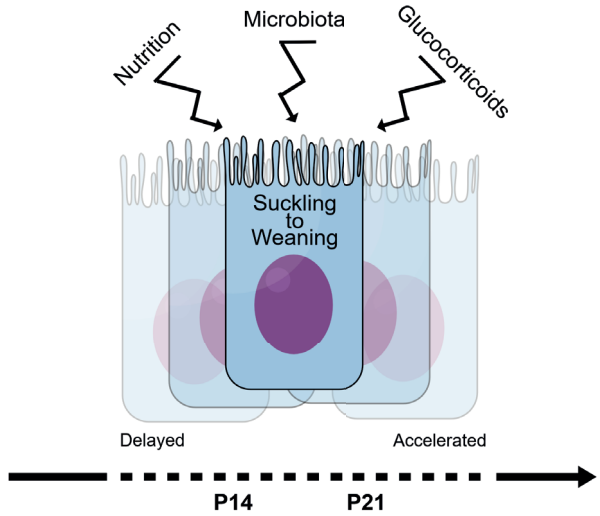

Figure 5 – The main three external factors capable of modulating the timing of intestinal epithelial maturation.
The type of nutrition and its components, the microbiota and its products, and hormones, such as glucocorticoids, can delay or accelerated intestinal maturation.

INTESTINAL MATURATION ON A DISH

The access to human intestinal material is, for evident ethical reasons, limited, thus most of the maturation studies have been performed in mice and rats. Even though the timing of intestinal development and maturation differs between humans and mice, the pattern is virtually the same [96]. Yet, research focussing on intestinal maturation has been limited by the lack of experimental models of the neonatal intestine. An overview of three main current models to study intestinal maturation and its limitations are highlighted hereafter (Figure 6).

Intestinal explants

The *in vitro* culture of segments of small intestine was the first method used to carefully study the suckling-to-weaning transition. These explants are open and kept for a short period of time (up to 3 or 4 weeks) submersed in culture medium. More than 30 years ago, researchers used explants to investigate the typical pattern of brush-border glycohydrolases expression and activity in the small intestine: decreased lactase and increased sucrase-isomaltase and trehalase around weaning [67, 68, 97-99].

Besides the extensive use of this method with mice and rat small intestine, explants have also been isolated from fetal human intestine [72, 76, 100-106]. Human fetal intestinal explants were used to study the pattern and mechanisms controlling brush-border glycohydrolases expression and to investigate the effects of different factors, including glucocorticoids, on the expression pattern of these glycohydrolases [72, 100, 101, 104]. As the only source of human fetal intestine is from authorized gestation termination clinics, the developmental stage of the studied material is restricted to between 10 and 20 weeks of gestation. Therefore, findings cannot be extrapolated to the maturation process occurring after birth, as the intestine up to 20 gestational weeks is still in an early maturation state. Nevertheless, research using human explants could corroborate many of the discoveries made in mice: brush-border glycohydrolases activity increases over time and this increase can be accelerated by glucocorticoids [72, 101, 104, 107].

Human and mouse explants have also been used in experiments demonstrating the intrinsic nature of intestinal epithelial maturation. Their transplantation into subcutaneous tissue or kidney of adult mice revealed that explants could mature despite the absence of luminal signs or hormonal status of the mother [61, 63, 75]. Nevertheless, the short duration of the viability of the tissue and the impossibility of studying the different mucosal layers separately are critical limitations of this model. Thus, explants are no longer the first choice for intestinal maturation studies.

Human Pluripotent Stem Cells

The limitations to access human late-stage fetal and neonatal material led to the recent use of human embryonic and induced pluripotent stem cells (hPSCs) as the start material to obtain human fetal small intestine. Researchers determined which factors are necessary for hPSCs to develop into endoderm/mesoderm and further into intestinal organoids [108-112]. Intestinal organoids are cultured in matrigel, an extracellular matrix that serves as a scaffold for the three-dimensional growth and expansion of the intestinal cells. These hPSCs-derived intestinal organoids have been used to study host-microbe interactions and epithelial barrier integrity upon exposure to environmental factors [113, 114]. However, such intestinal organoids derived from hPSCs have been shown to be more similar to the fetal intestine (12 to 16 gestational weeks) than to the late fetal/postnatal intestine [109, 110, 113, 114]. However, when transplanted under the kidney capsule of mice, these hPSCs-derived intestinal organoids acquire mature intestinal characteristics: crypt-villus structures, Paneth cells, and adult brush-border enzymes [109, 110, 114]. Effort has also been made to achieve maturation *in vitro* using specific culturing conditions [111, 115]. Nonetheless, these organoids are not yet able to mimic the late fetal and early postnatal intestine completely. Thus, they still present strong limitations for the study of early life factors affecting intestinal maturation.

Fetal organoids

Isolation of primary mouse IEC's to grow fetal organoids has been used to study the developmental progenitors of adult stem cells [25, 116]. These fetal organoids give rise to two different types of structure, spheroids, and organoids. The percentage of spheroids has been shown to be inversely related to the age of the intestine: while IEC's isolated from E14 intestine give rise to almost 100% spheroids, E18 IEC's generate more than 60% organoids, and at P15 IEC's exclusively form organoids. This indicates that later developmental stages originate lower number of spheroids and higher number of organoids. Although fetal intestinal spheroids/organoids were described as incapable of maturing over time, they expressed gastric markers, suggesting that the acquisition of specific intestinal markers did not yet occur. This is in accordance with the developmental stage used for spheroid/organoid isolation, as at E14 intestinalization has not yet occurred [24]. Recently, researchers have also started to study human intestine maturation using fetal organoids isolated from 11 to 22 gestational weeks human intestine [116-118].

Whether fetal organoids isolated from mouse intestine at late developmental stage can indeed mature *in vitro* and recapitulate suckling-to-weaning transition will be described in the second chapter of this thesis.

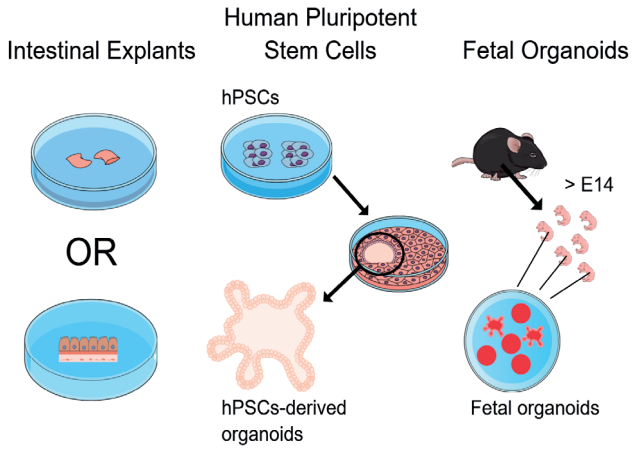

Figure 6 – Experimental models of intestinal maturation.
Intestinal explants were the first *in vitro* method to study intestinal maturation but due to its short viability they are less used nowadays. The directed differentiation of human pluripotent stem cells into intestinal organoids is suitable for the investigation of the early stages of intestinal maturation. Organoids can also be grown from primary intestinal epithelial cells isolated from mouse fetuses and cultured in Matrigel.

A VULNERABLE TIME

The neonatal stage is a vulnerable period. The intestinal epithelium is still immature, the intestinal barrier is leaky, the immune system is actively kept naive to tolerate the exposure to microbial colonization, and the gut microbiota is less diverse. At weaning, the intestinal epithelium becomes mature, the intestinal barrier decreases its permeability, the adaptative immune system begins to respond to the striking increase in dietary antigens from the solid food and microbial antigens from the expansion in microbial diversity [119]. Early life events occurring during this neonatal period up until weaning will be imprinted into how the gut develops and shape its long-life health. This creates a defined "window of opportunity" during which the intestine is exposed to early life factors, such as the type of milk feeding, infections, and antibiotics, that can affect not only its development but also influence health later in life [119-121]. This perinatal programming concept was first demonstrated by Barker in 1989 that showed that infant weight was correlated with death from ischemic heart disease [122]. In the following decades, the development of several chronic non-communicable diseases, many related to the gut, has also been associated with perinatal factors [120, 123-126].

Early life events have a strong impact on short- and long-term health, making this phase one of the most important for disease prevention [119-121]. An external factor that is commonly present in early life is antibiotics. Its use during early life has been linked with the development of diseases both upon treatment as well as later in life. Understanding how antibiotics can affect intestinal development and maturation is essential in order to limit their consequences and find alternatives.

ANTIBIOTICS SAVE BABIES

The first antibiotic was discovered less than 100 years ago and its medical use and mass production started only 20 years later. Rapidly, different antibiotic types were found and new classes were introduced. This life-saving drug revolutionized health care in the world. Antibiotics became not only a treatment drug but also a prophylactic medication. Moreover, their use to enhance animal growth for meat production turned them into an unavoidable presence on our plates. The widespread use of AB led to the increased development of several gut-related diseases resulting from antibiotic-resistant bacteria and commensal microbiome disruption. Today, global antibiotic consumption keeps increasing, particularly driven by the economic growth of low- and middle-income countries [127]. Researchers try to find new alternatives for antibiotics, but to fully replace their use in health care is decades away. It is crucial that we not only decrease the

use of AB but also reduce the incidence of associated pathologies. We must, therefore, do our best to understand how AB contributes to the development of such diseases.

In infants and children, the use of AB is a common practice, making them the most frequently prescribed medication in the Western world [128]. Each year in Europe, more than 10% of children are given AB, while in the USA this percentage is as high as 25% [128, 129]. The frequent use of AB in infants and children is linked with the high prevalence of infectious diseases during early life. These include infections of the respiratory tract, ear, skin, and gastrointestinal system [130]. Besides, AB are not only used for the treatment but also for the prevention of infections. For example, AB are widely prescribed to preterm neonates suspected of early onset neonatal sepsis. Considering that more than 10% of global births are preterm, every year there are almost 15 million preterm babies [131].

The main AB classes used in infants and children are (Figure 7):

- Beta-lactams, a bactericidal class of antibiotics that acts by interrupting the bacterial cell wall synthesis, resulting in cell lysis [132]. Examples of this class are amoxicillin, penicillins, and ampicillin. β-lactams affect both gram-positive and gram-negative bacteria and are prescribed when a systemic infection is suspected or confirmed [133-136].
- Glycopeptides have bactericidal activity against most organisms and bacteriostatic effect on enterococci; they bind tightly to the D-alanyl-D-alanine portion of the cell wall precursors, thereby interfering with bacterial cell wall synthesis [137]. This leads to the activation of bacterial autolysins that destroy the cell wall by lysis. This class acts on gram-positive bacteria. In neonates and children, the glycopeptide vancomycin is administered for the treatment of *Clostridium difficile*, *Staphylococcal enterocolitis*, and *Staphylococcus aureus* infections [133, 138-143].
- Nitroimidazoles are converted into metabolites that impede intracellular bacterial DNA synthesis and are, therefore, bactericidal [137]. Metronidazole is one example of this class, it has a broad spectrum and affects anaerobes. It is easily absorbed in the gastrointestinal tract, particularly in the duodenum and beginning of the jejunum, and it is prescribed, for example, in case of gastrointestinal complications, frequently in combination with amoxicillin [139, 144-147].
- Aminoglycosides affect the elongation step of the bacterial protein synthesis inhibition by binding irreversibly to the aminoacyl site of 16S ribosomal RNA within the 30S ribosomal subunit, leading to the misreading of the mRNA and production of nonsense and toxic proteins [148, 149]. Aminoglycosides are commonly prescribed due to their broad spectrum, affecting aerobic gram-positive

and gram-negative bacteria. As uptake of aminoglycosides by bacteria requires an active electron transport chain, anaerobic ones are intrinsically resistant to aminoglycosides. Examples of this class are amikacin, gentamicin, neomycin, streptomycin, and tobramycin. Although commonly used in neonates and chil-

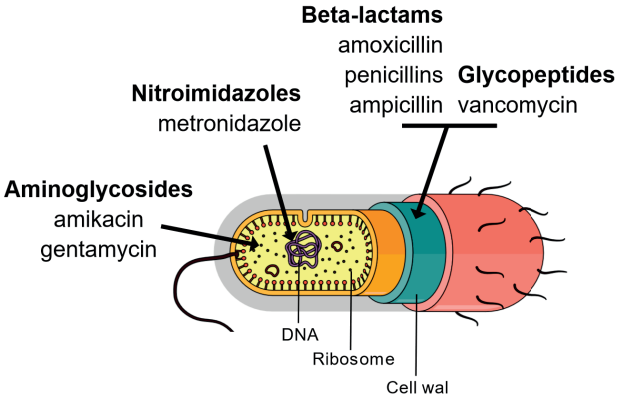

Figure 7 – Mechanisms of action of commonly used antibiotics in early life.
Beta-lactams and glycopeptides block the synthesis of the bacterial cell wall. Nitroimidazoles impede the bacterial DNA synthesis. Aminoglycosides target ribosomes and interfere with the bacterial protein translation.

dren, aminoglycosides are poorly absorbed in the intestine and must be given intravenously or intramuscularly.

The prolonged use of AB in early life is associated with several diseases, both short-term and long-term (Figure 8). AB do not only target bacteria but can also interact with the host cells in two different ways. On one hand, they can cause the disbalance of the commensal gut bacteria, allowing the proliferation of invasive strains and cause reduction of bacterial diversity. Consequently, gut cells respond to these differences, ultimately leading to the onset of different pathologies [123, 150-157]. On the other hand, AB can directly act on the host IECs cells, affecting their gene/protein expression and metabolism [158-160]. Yet, little attention has been given to these non-antimicrobial effects of antibiotics which can influence IECs, independently of the microbiota.

Human studies on the effect of antibiotics on the intestinal epithelium are complex, a consequence of the many confounding effects and limited untreated control subjects, as usually healthy subjects are used which will have a different medical background compared to the treated subjects. Therefore, studies in mice have contributed to the first steps in understanding how AB affect host cells. However, most of these studies have been performed in adult mice [156, 161, 162]. As described above, the adult intestine

is considerably different from the neonatal intestine. Thus, findings do not completely reflect the effect of early life AB treatment on the immature neonatal intestine.

In literature, the effects of AB on the gut have mainly been studied using a combination of AB within four classes: β-lactam (usually ampicillin or amoxicillin), glycopeptide (vancomycin), nitroimidazole (metronidazole), and aminoglycoside (amikacin, gentamycin, neomycin, streptomycin) [152, 158, 163-166]. These classes are also commonly used in neonates and children [129, 133, 138, 139, 141-143, 146, 167-172]. As aminoglycosides

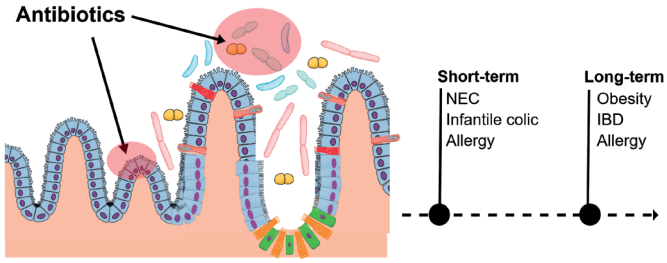

Figure 8 – The short-term and long-term effects of antibiotic treatment in early life.
Antibiotics can affect the intestinal epithelium indirectly, as a result of changes caused to the intestinal microbiome, or by direct action on the epithelial cells. Antibiotic treatment during the first weeks of life has been associated with neonatal conditios, such as necrotizing enterocolitis (NEC), infantile colic, and allergy, and with the development of diseases later in life, including obesity, inflammatory bowel disease (IBD), and allergy.

are poorly absorbed by the gastrointestinal tract, the work in this thesis excluded this category of AB and focused on the combination of the other 3 most commonly used AB: amoxicillin, vancomycin, and metronidazole.

TWO SIDES OF THE SAME COIN

Infants and children that received AB in early life have a higher risk of developing certain conditions during infancy and childhood, such as infantile colics [173], and allergic diseases [174, 175]. Besides, the risk of developing NEC in preterm neonates, due to the immature state of their intestines [176], has been shown to possibly increase with AB treatment [177-179]. Although the causes of NEC are still unknown, it manifests as intestinal inflammation and bacterial invasion of the intestinal epithelium, leading to cell death and intestinal perforation [21]. It is a devastating disease, with long-term sequelae and mortality rates between 10% to 50%. Still, some evidence seems to indicate that the association between AB use and NEC development could be related to the type of antibiotic used [166, 180].

Early life exposure to AB can also contribute to the development of allergies [174, 175, 181], obesity [150, 182-185], and IBD [120, 181, 186-190] later in life. Recent evidence demonstrates that the increased incidence of these diseases is a result of intestinal microbiota disturbance during a specific neonatal time window, which affects the development of the immune system and leaves the organism more sensitive to immunopathologies in adulthood [191-197]. Al Nabhani *et al.* established that antibiotics in early life prevent the "weaning reaction" (an essential immune reaction to the colonization of the gut by commensal microbiota that occurs during weaning) resulting in a higher susceptibility to colitis, allergy, and cancer in mice [191]. Nevertheless, changes in IECs during the suckling-to-weaning transition and at weaning might also persist until adulthood, for example, if epigenetic imprinting is affected [65]. Furthermore, the timing of AB treatment also seems important in determining their effects. For example, AB given during the first two weeks of life impair weight gain while AB exposure later in the first years of life are associated with increased weight gain [198, 199]. Finally, the increased risk of childhood allergy observed after AB treatment during the first week of life is independent of additional AB in the first two years of life, further demonstrating the importance of the timing of AB treatment [200].

AN INDEPENDENT PROBLEM

Researchers have mainly connected the effects of AB on gut health and development to the changes caused by AB in the gut microbiome. However, a few studies have suggested that AB also have non-antimicrobial effects, i.e. effects that are independent of differences in the microbiome, resulting from the direct action of AB on the intestinal cells. One of the first pieces of evidence that antibiotics can interfere directly with gut cells was demonstrated when germ-free mice, which are depleted of all bacteria and are kept in sterile conditions, were treated with antibiotics [158]. Gene expression analysis of the ileum revealed that several genes were affected by the AB treatment, although no microbiome is present in these mice. Moreover, 617 of these differentially expressed genes were similarly affected in conventionally housed mice treated with AB. When examining these genes, it was shown that most of these genes are expressed in crypts and villi epithelial cells and code for proteins performing ribosomal and mitochondrial functions, such as electron transport chain, oxidation-reduction, ATP biosynthesis, and cellular and mitochondrial ribosomes. These effects are similar to the mechanism of antibiotic action on bacteria, as many antibiotics interfere with protein synthesis by targeting ribosomes [201] or/and affect their metabolic state [202]. The similarity between the targets of AB in bacteria and in epithelial cells, especially the ones related to mitochondria, is supported by the endosymbiotic theory [203]. This evolutionary theory proposes

that the eukaryotic cell evolved from the symbiotic relationship between two bacteria, one engulfed by the other. The resemblance of bacteria and mitochondria is one of the strongest pieces of evidence of this theory. Indeed, treatment of the intestinal epithelial cell line Caco2 with the β-lactam ampicillin, the fluoroquinolone ciprofloxacin, and the aminoglycoside kanamycin was shown to increase protein carbonylation (protein oxidation promoted by ROS) and lipid peroxidation (oxidative degradation due to free radicals) [160]. Furthermore, an increase in mitochondrial superoxide production and extracellular H_2O_2 release was detected. Ampicillin also inhibited electron transfer chain (ETC) complexes (individually isolated); decreased mitochondrial membrane potential, ATP levels, and overall metabolic activity, induced shorter and truncated mitochondria, and reduced basal respiration and maximal respiratory capacity. Moreover, vancomycin treatment of a porcine renal tubular epithelial cell line for 24h has been shown to lead to depolarization of the mitochondrial membrane potential, release of cytochrome *c* from the mitochondria into the cytosol, increase in ROS production, reduction in the activity of the antioxidative enzyme superoxide dismutase, increase in mitochondrial O^{2-} production, and inhibition of mitochondrial complex I activity [204]. Additionally, vancomycin was found to alter mitochondria activity, leading to downregulation of antioxidant genes and consequent upregulation of HMOX1, a gene associated with cellular oxidative damage [205].

OUTLINE OF THE THESIS

At birth, the pivotal intestinal functions are not completely developed and infants are born with an immature gut that reaches maturity around the second birthday, after the complete switch in diet from milk to solid food. Changes in the normal development and maturation of the intestine during this postnatal period affect not only the neonatal intestine but also influence health later in life. Several studies in humans and mice have shown the association between disturbed intestinal development and the onset of various diseases. However, the research into the mechanisms behind these associations has been held back by the limited access to intestinal material at this stage of life and the lack of experimental models of the neonatal intestine. This prompted us to develop an *in vitro* model using mouse fetal intestine organoids to study intestinal maturation. The establishment of this organoid *in vitro* model is characterized in **chapter 2**. In this chapter, a proof-of-principle experiment demonstrates that these mouse fetal intestine organoids can be used to study the influence of external factors on intestinal maturation. In **chapter** 3, a step-by-step video protocol of how to culture and analyze mouse fetal intestine organoids is described.

Infants are more vulnerable to infections than adults. Their immune system has a limited capacity to protect the organism from bacteria, resulting in more frequent infections. Consequently, early life antibiotics are intensively used in infants for the treatment of confirmed or suspected infections. However, prolonged antibiotic exposure early in life has been associated with adverse outcomes in the neonatal period and later in life. Two reasons explain why many of these pathologies are related to the gut. First, the neonatal intestine at birth is still immature. Second, the establishment of commensal bacteria in the intestine, necessary for its normal functioning, also takes place in early life, so antibiotic treatment during this time frame will interfere with the amount and diversity of bacteria colonizing the intestine. Thus, antibiotics given during such a critical moment will greatly influence intestinal health. In **chapter 4**, we investigate the effects of early life antibiotics on postnatal intestinal maturation and functioning, using both neonatal mice and mouse fetal intestinal organoids. The fetal organoid model is used to determine which of the effects result from the direct action of the antibiotics on the intestinal epithelial cells, independently of changes on microbiome or other intestinal cell types. **Chapter 5** identifies the consequences of antibiotic treatment that persist in the adult small intestine and examines whether new effects are detected. Finally, all findings of this thesis and future perspectives are discussed in **chapter 6**.

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2

Mouse Fetal Intestinal Organoids: New Model To Study Epithelial Maturation From Suckling To Weaning

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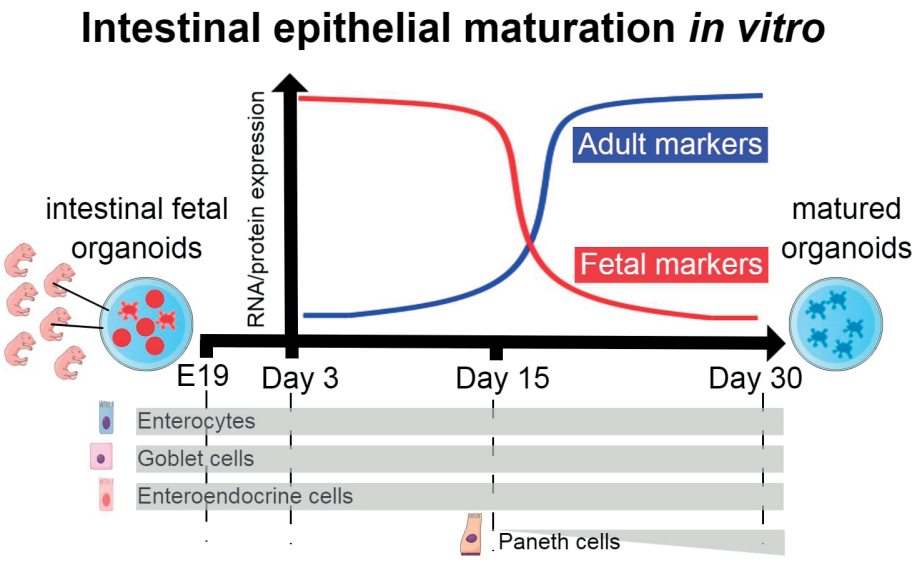
EMBO reports, 2019, 20: e46221

ABSTRACT

During the suckling-to-weaning transition the intestinal epithelium matures, allowing digestion of solid food. Transplantation experiments with rodent fetal epithelium into subcutaneous tissue of adult animals suggest that this transition is intrinsically programmed and occurs in the absence of dietary or hormonal signals. Here, we show that organoids derived from mouse primary fetal intestinal epithelial cells express markers of late fetal and neonatal development. In a stable culture medium, these fetal epithelium derived organoids lose all markers of neonatal epithelium and start expressing hallmarks of adult epithelium in a time frame that mirrors epithelial maturation *in vivo*. *In vitro* postnatal development of the fetal derived organoids accelerates by dexamethasone, a drug used to accelerate intestinal maturation *in vivo*. Together, our data show that organoids derived from fetal epithelium undergo suckling-to-weaning transition, that the speed of maturation can be modulated, and that fetal organoids can be used to model the molecular mechanisms of postnatal epithelial maturation.

KEYWORDS

Intrinsic intestinal epithelial maturation / mouse fetal organoids / suckling-to-weaning transition / brush border enzymes



INTRODUCTION

At birth, the mouse small intestinal epithelium consists of a single layer of epithelium that covers finger-like projections into the intestinal lumen called villi. At this time, the villi are populated with three intestinal cell types: the enterocyte, goblet cell and enteroendocrine cell. Proliferating epithelial cells are localized at the base of the villi in so-called “intervillus pockets” [1]. In the first month after birth, the epithelium undergoes major structural and functional changes. The most apparent structural changes occur around two weeks after birth, when crypts form at the base of the villi and the Paneth cells start to populate the bottom of the crypts [2,3]. Concurrently, major functional changes are initiated in a process called suckling-to-weaning transition, which is completed within two following weeks. This transition consists of a number of highly specific enzymatic and metabolic changes that allow diet change from milk that is rich in fat and has lactose as a major carbohydrate, to solid food that is rich in complex carbohydrates [4]. Many of the major changes occur at the epithelial brush border, which expresses various proteins involved in the digestion of food, such as enzymes needed for processing of carbohydrates. More specifically, in the first two postnatal weeks the principal carbohydrate is lactose, and its digestion is dependent on the enzyme lactase phlorizin hydrolase (Lct) [5]. Around postnatal day 14 in mice (P14), the adaptation to digest complex carbohydrates from solid food is initiated. This is accompanied with gradual increase in expression of the brush border disaccharides sucrase-isomaltase (Sis) and trehalase (Treh). Expression levels of these enzymes rise rapidly to adult levels in the third postnatal week [4,6].

One of the key metabolic changes during postnatal development involves arginine biosynthesis. Arginine is a semi-essential amino acid that is only present in limited amounts in milk and therefore synthesized in the neonatal enterocytes [7]. Argininosuccinate synthetase 1 (Ass1) is the rate-limiting enzyme in arginine biosynthesis and in mice exclusively expressed in the first two postnatal weeks. During the suckling-to-weaning transition, epithelial cells lose the expression of Ass1 and switch to expressing arginase 2 (Arg2) allowing catabolism of arginine, which is abundantly present in solid food [7,8]. Another characterized occurrence of the suckling-to-weaning transition in mice is loss of expression of the neonatal Fc receptor for immunoglobulin (FcRn) [9,10]. Neonatal intestinal epithelium expresses high levels of FcRn, which mediates the transfer of maternal IgG from the milk across the intestinal epithelial membrane to facilitate passive immunity. In addition, loss of cathelicidin-related antimicrobial peptide (CRAMP) during the suckling-to-weaning transition of mouse intestinal epithelium has been described as well [11].

Transplantation studies of fetal intestinal segments into subcutaneous tissues of nude adult mice revealed that these segments developed normally in the absence of luminal signals [12-14]. These experiments established that information needed for appropriate intestinal epithelial development, including the suckling-to-weaning transition, is driven by a genetic program that is intrinsic to the intestinal mucosa and specified in early development.

We and others have previously shown that the intestinal transcription factor Blimp-1 is selectively expressed in the mouse intestinal epithelium during embryonic and postnatal development, and that its expression is lost at the suckling-to-weaning transition [15,16]. Conditional deletion of Blimp-1 from the mouse intestinal epithelium resulted in an adult type epithelium at birth, with complete absence of ultrastructural and molecular characteristics of postnatal phase of development and severe growth impairment and death of newborn pups [15,16]. These data showed that Blimp-1 in the mouse intestinal epithelium is a critical driver of the postnatal epithelial phenotype and that its loss of expression in the third postnatal week is likely required for maturation from neonatal to adult epithelium.

The factors driving Blimp-1 expression in the first two weeks and its loss of expression in the third postnatal week are not known. The suckling-to-weaning transition might be completely intrinsically regulated, but epithelial gut maturation can to some extent be modulated by hormonal status and extrinsic luminal signals like microbiota and nutrition. Changes in endogenous and exogenous circulating hormones in the developing neonate, such as glucocorticoids, can precociously induce intestinal maturation *in vivo* [17,18]. Luminal signals, such as microbiota, regulate developmental-dependent expression of epithelial glycosyl transferases, enzymes necessary for glycosylation of epithelial-specific proteins during gut maturation [19]. Finally, dietary factors such as growth factors, human milk oligosaccharides and hormons that are present in human milk have been shown to influence gut growth and maturation in cell lines and/or rodent models [20,21].

It has been an ongoing discussion to which extent, and which specific aspects of suckling-to-weaning transition are intrinsically programmed. Here, we use cultures of primary fetal epithelial cells to examine if the epithelial suckling-to-weaning transition also occurs *in vitro*, in a stable culture medium and in absence of stromal cells.

RESULTS AND DISCUSSION

Primary mouse fetal intestinal epithelium matures *in vitro*

To study whether the intestinal epithelium matures and undergoes the suckling-to-weaning transition *in vitro*, intestinal epithelium from developmental stage E19 was chosen as starting material for the organoid cultures. Organoids were cultured for one month, in a stable culture medium, following the passage scheme and harvesting of organoids on the same day after each passage (Fig 1A). As isolated intestinal epithelial cells need 24 to 48h to establish *in vitro* growth, this developmental stage translates *in vitro* just prior to birth. We performed genome wide gene expression analyses on fetal and adult organoids at day 3 and 30 of culture and mouse intestinal tissues at birth (day 0) and adult (day 42). Principal component analysis (PCA) of this multi-dimensional dataset revealed that 4 clusters can be distinguished based on gene expression profiles: 1) fetal organoids day 3, 2) fetal organoids day 30 toghether with adult organoids (day 3 and 30), 3) fetal tissue, and 4) adult tissue (Fig 1B). Along the first component (PC1 34%), the organoids (epithelium) are clearly separated from the whole tissue, indicating that the gene expression profile of organoids differs substantially from intestinal tissues. Along PC2 (PC2 16.2%) the day 3 fetal organoids separate from day 30 fetal and day 3 and 30 adult organoids, as is also the case for fetal and adult tissue. Of note, no significant difference in the global gene expression profile between day 30 fetal organoids and day 3 or 30 adult organoids assessed by Pearson correlation is observed (Figs EV1A and B). The direction of separation along PC2 for organoids and tissue is the same, suggesting that the maturation state contributes to this separation.

To assess differences and similarities between organoids and tissues and to examine these differences relative to those that exist when comparing fetal intestines and adult intestines, we performed a differential gene expression analysis. Out of 178 genes (Table EV1) that were upregulated 4-fold or more in fetal organoids cultured for 30 days, 115 genes (65%) were in common with genes upregulated in the adult tissue (Fig 1C). Similarly, 105 out of 310 genes (35%) overlapped between downregulated genes in fetal organoids cultured for 3 days and fetal tissue (Fig 1D). Heat map of the top 76 upregulated genes after 30 days of E19 organoid cultures clustered with adult (day 42) tissue (Fig 1E). Moreover, the majority of the most downregulated genes in 30 day-old fetal organoid cultures were similarly expressed in fetal tissue. Additionally, comparison of our fetal organoid dataset to previously published transcription profiles of primary isolated intestinal epithelial cells of neonatal versus adult mice (GEO GSE35596) [22,23] revealed that 200 upregulated genes in neonatal mouse epithelium were significantly enriched in the fetal organoids cultured for 3 days, whereas 200 downregulated genes correlated with fetal organoids after 30 days of culture (Figs EV1C and D). We next performed Ingenuity

Pathway Analyses (IPA) using as input the list of differentially expressed genes between day 3 and day 30 fetal organoid cultures. Predominant changes in canonical pathways involved metabolic alterations indicative for epithelial maturation that is associated with a change in diet from mother's milk to solid food (Fig 1F). Together, these analyses suggest that fetal organoids mature over time and undergo the suckling-to-weaning transition *in vitro***.**

In vivo maturation process of mouse intestinal epithelium

We first examined the intestinal epithelial maturation *in vivo* in detail, using a panel of maturation markers that are described in literature as markers for fetal/neonatal, suckling-to-weaning, and adult epithelium. With this approach, we aimed to obtain a standard for temporal comparison with the *in vitro* maturation process of the E19 fetal organoids. In the fetal phase (E18.5), we observed a strong expression of the neonatal enzyme Argininosuccinate synthetase 1 (Ass1) (Figs EV2A and D), transcription factor Blimp-1 (Figs EV2B and E) and neonatal Fc receptor (FcRn) (Fig EV2F) throughout the whole epithelium. Histological assessment of tissues from the first two postnatal weeks (P7.5 and P14) showed that expression of these markers gradually disappeared from the proliferative intervillus regions but remained in the differentiated cells of the villi. In the adult gut (P42), expression of Ass1 was completely lost (Fig EV2A), whereas Blimp-1 was restricted to a limited number of cells at the villus tips (Fig EV2B). *In vivo*, Lct was highly expressed in rodent neonatal epithelium and declined after weaning, however still present in the adult intestine, mainly in the jejunum (Figs EV2C and G) [24,25]. Activity of Lct peaked at weaning, yet it remained present in adult albeit at lower level (Fig EV2H). Although Lct expression pattern differed from Ass1 and Blimp1, it is characteristic for neonatal development and is associated with milk diet [24,25].

In contrast to the neonatal enterocyte markers, the adult α-glucosidases sucrase-isomaltase (Sis) (Figs EV3A and C) and arginase-2 (Arg2) (Figs EV3B and D) were absent from the small intestine epithelium until the second week after birth, around the suckling-to-weaning transition, when a gradual increase of both enzymes was observed exclusively in the villi. The same pattern was observed for trehalase (Treh) (Fig EV3E). These results were confirmed at enzyme activity level (Figs EV3I-L). In addition, expression of the Paneth cell markers lysozyme-1, (*Lyz1)* and α-defensins (*Defa1, Defa5)* were detected from day 14 onwards (Figs EV3F-H). This correlates with the maturation of this secretory cell type at two weeks after birth, concurrently with the development of the crypts. The *in vivo* maturation pattern described here was subsequently used and compared with the time course of maturation of the fetal small intestinal organoids *in vitro* as described below.

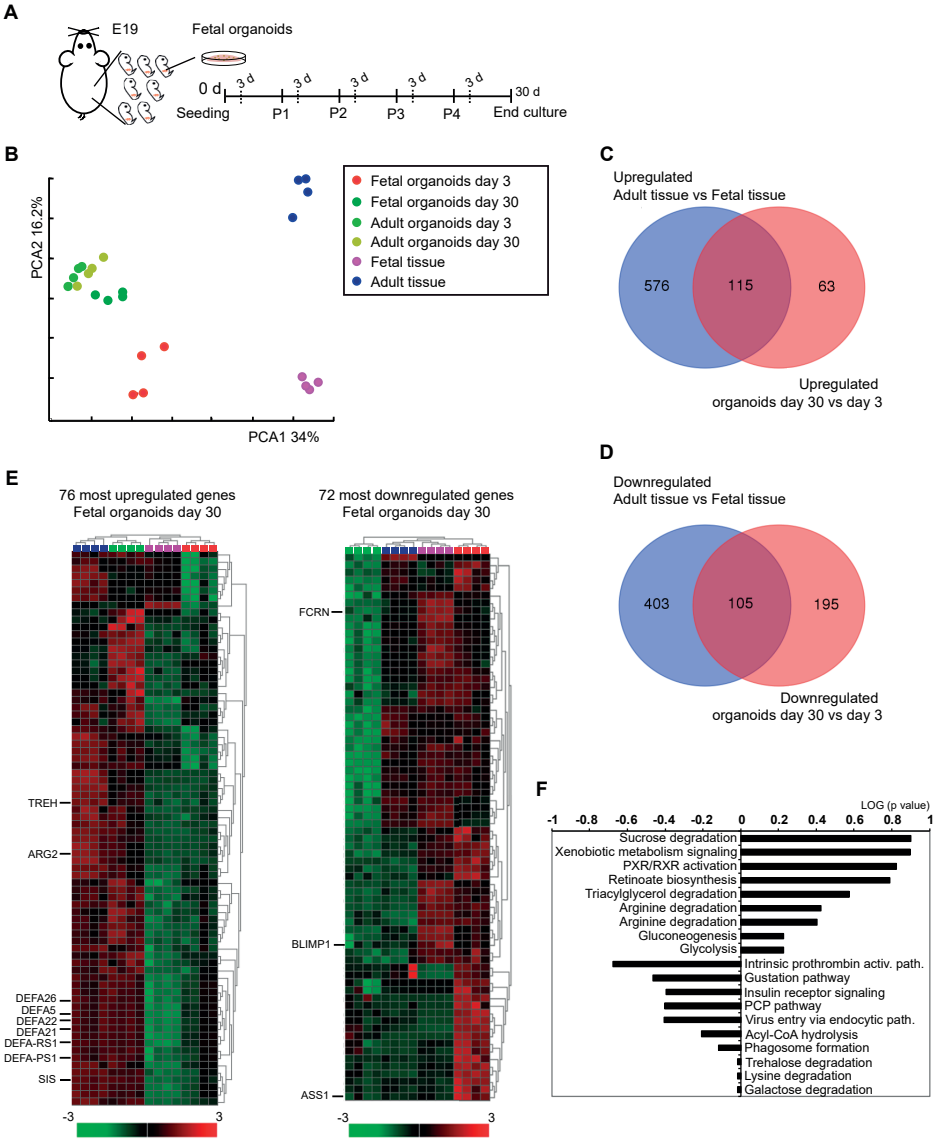


Figure 1 – Gene expression analyses of E19 organoids at early and late culture time points.

A Fetal organoids isolated from fetal intestine at embryonic day 19 were cultured for 30 days in ENR medium and analysed 3 days after indicated passage.

B PCA was conducted on global gene activity in mouse fetal tissue at day 0 and 42, mouse E19 organoids at day 3 and 30 of culture, mouse adult organoids at day 3 and 30 of culture (n=4 independent intestinal tissue specimens and n=4 independent organoid cultures).

C,D Lists of differentially expressed genes between adult and fetal tissue, day 3 and 30 organoids were generated for **C** up and **D** down regulated genes. Results are shown as Venn diagram.

E Curated heatmaps of top 76 up and 72 down regulated genes. Highlighted genes were chosen based on biological interest. The coloured bar represents the expression level from low (green) to high (red).

F The most significantly changed canonical pathways between fetal organoid at day 3 and 30 of culture.

Small intestinal fetal organoids mature and recapitulate suckling-to-weaning transition *in vitro*

We first analyzed the expression pattern of the fetal/neonatal enterocyte markers in the cultured fetal organoids by qRT-PCR. Both *Ass1* and *Blimp-1* were expressed during the first week of culture and nearly absent after three weeks (Figs 2A and B). Similarly, FcRn and *CRAMP* (Figs 2C and EV4A) followed the same expression pattern. Likewise, Lct (Fig 2D) expression was similar to the *in vivo* expression pattern (Fig EV2G). In contrast, markers of the suckling-to-weaning transition and adult intestine *Sis* and *Treh* were only detected in organoids as of two weeks of culture (Figs 2E and F). *Arg2* was expressed at one week of culture (Fig 2G) and progressively increased thereafter. Development of a functional brush border was confirmed on enzyme activity level (Figs 2H-L). Comparing the *in vitro* maturation from suckling-to-weaning with the *in vivo* maturation process revealed that the time frame of epithelial maturation is similar between the fetal organoids *in vitro* and the intestinal tissue *in vivo* (compare Figs 2A-D to EV2 for neonatal markers and Figs 2E-G to EV3 for suckling-to-weaning/adult markers). Previously it has been reported that different fetal intestinal segments, i.e. proximal versus distal, generate different organoids [26]. We therefore separated proximal and distal parts of E19 intestinal epithelium and studied the *in vitro* maturation course of the fetal organoids originating from these segments (Appendix Figure S1). Although relative expression levels of some of the maturation markers differed among the segments, the timing and the course of maturation was similar (Appendix Figure S1).

Lgr5, the best described adult intestinal stem cell marker under homeostatic condition, is expressed by intercolumnar cells residing inbetween the Paneth cells. We next investigated the presence of *Lgr5* expressing cells throughout the course of our culture by means of RNa scope *in situ* hybridization complemented with immunofluoresce for Lyz1, a marker for Paneth cells (Appendix Figure S2). At the start of E19 culture, *Lgr5* was expressed at low levels throughout the organoid epithelium and Paneth cells were absent. Coinciding with the appearance of Paneth cells, *Lgr5* signals increase and become confined to the crypt region. Finally, Paneth cell-specific markers *Lyz1*, *Defcr1* and *Defcr5* were detected at two weeks of culture (Fig EV4B-D), again similar to the intestinal tissue *in vivo* (Fig EV3F-H). Together, these findings demonstrate that fetal intestinal organoids, when cultured *in vitro,* follow an intrinsic epithelial maturation pattern characteristic for the *in vivo* maturation program in a similar time frame.

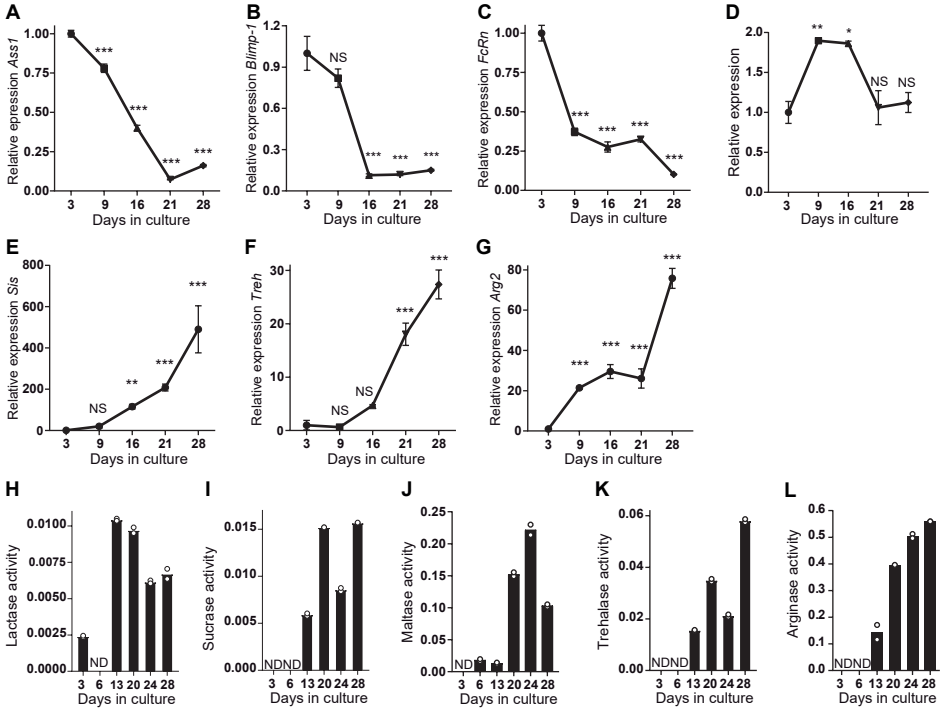


Figure 2 - **Small intestinal fetal organoids recapitulate suckling-to-weaning transition *in vitro*.**
A-G Real-time qPCR analysis of fetal organoids cultured for one month showing decrease in relative expression of indicated neonatal markers **A** *Ass1*, **B** *Blimp-1*, **C** *FcRn* and **D** *Lct* and increase of the adult markers **E** *Sis*, **F** *Treh* and **G** *Arg2* (n=3 individual wells from single organoid culture (see material and methods); experiment was repeated in four to eight independent organoid cultures with similar results). **H-L.** Enzyme activity assay of fetal organoids for **H** Lactase, **I** sucrase, **J** maltase, **K** trehalase and **L** arginase. Activity is given in µM glucose/µg protein·min^{-1} (experiment was generated from single organoid culture (see material and methods) and repeated in 3 independent organoid cultures with similar results). Data information: Data are presented as mean ± SEM. ND not detected, *p<0.05, **p<0.01, ***p<0.001, NS not significant, relative to expression or activity level at day 3 (one-way ANOVA).

Fetal organoids resemble adult organoids after one month in culture

At one month after birth the mouse intestinal epithelium *in vivo* reaches its adult functional state. We cultured E19 fetal and adult intestinal organoids simultaneously for one month (Fig EV4E). Indeed, at day 30 of culture, fetal and adult organoids expressed similar levels of the maturation markers (Figs 3A-G). Importantly, expression levels of neonatal markers remained absent in adult organoids, while adult markers were stably expressed throughout the 30 days of culture. This was further confirmed at enzyme activity level for all enzymes analyzed (Figs 3H-L). Moreover, except for the Paneth cells which became evident as of 14 days of culture, the main epithelial cell types, i.e. enterocytes, enteroendocrine, and goblet cells, were present in the fetal cultures from the start up untill 30 days of culture (Appendix Figure S2 and S3). Expression levels of markers for enterocytes, enteroendocrine and goblet cells were relatively stable over

time in fetal organoids (Figs EV4F-H). This demonstrates that fetal organoid maturation *in vitro*, as described here, is not a consequence of the long term culturing process of organoids. These findings, alongside with virtually no difference in transcription profiles in prolonged culture of adult organoids (Figs 1B and EV1A), impose intrinsic transition from fetal to adult features *in vitro* and exclude prolonged culture as a contributor to this process.

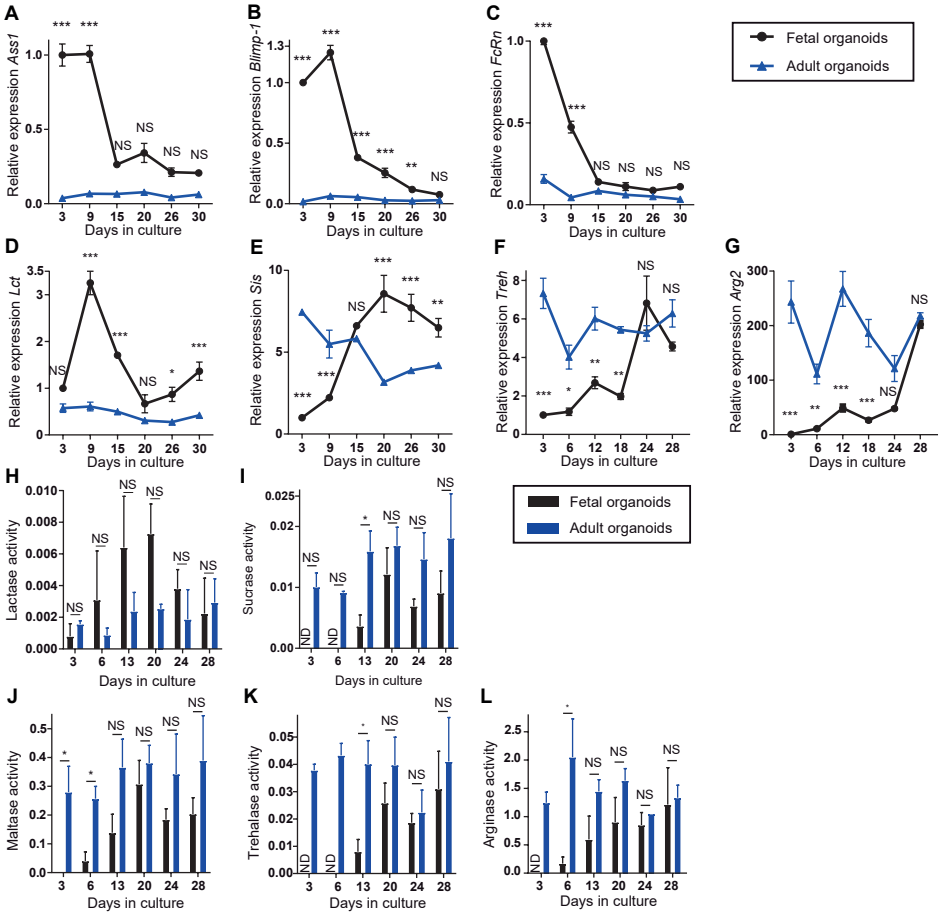


Figure 3 – **Fetal organoids resemble adult organoids after one month in culture**.

A-G Relative expression detected by Real-time qPCR in fetal organoids ( ) and adult organoids ( ). Neonatal markers **A** *Ass1*, **B** *Blimp-1*, **C** *FcRn* and **D** *Lct* in fetal organoids cultured for one month compare to levels adult organoids. Mature markers **E** *Sis*, **F** *Treh* and **G** *Arg2* increase in fetal organoids to adult levels (n=3 individual wells from single organoid culture (see material and methods); experiment was repeated in four independent organoid cultures with similar results). **H-L** Enzyme activity assay of fetal ( ) and adult ( ) organoids. **H** Lactase activity is higher in fetal organoids decreasing to adult levels at day 28, while **I** sucrase, **J** maltase, **K** trehalase and **L** arginase increase to adult levels. Activity is given in µM glucose/µg protein·min^{-1} (n=3 independent organoid cultures).

Data information: Data are presented as mean ± SEM. ND not detected, *p<0.05, **p<0.01, ***p<0.001, NS not significant, in A-G between fetal and adult organoids and in H-L relative to adult levels (t-test).

Dexamethasone accelerates maturation of fetal organoids *in vitro*

Extrinsic factors have been described to modulate the timing of intestinal epithelial maturation. For example, changes in dietary composition, microbiome and circulating glucocorticoids influence the expression of epithelial-specific enzymes enzymes [17,27,28]. We chose to use the synthetic glucocorticoid dexamethasone as an example of a factor that can influence the maturation process associated with the suckling-to-weaning transition of the mouse intestinal epithelium. Dexamethasone is described to increase epithelial proliferation and subsequently replace neonatal enterocytes by 'adult-like' epithelium, thereby leading to the precocious expression of the enzymes Treh and Sis [6,29,30]. To verify whether a similar effect could be observed in our *in vitro* culture, we treated fetal organoids daily with dexamethasone. Gene expression analysis of dexamethasone treated fetal organoids showed a rapid decrease of *Blimp-1* (Fig 4A) accompanied by an increase of *Sis* (Fig 4B) to adult levels two weeks earlier than the control condition. This was confirmed at enzyme level, where the enzymes sucrase and maltase exhibited a significant increase in activity after dexamethasone treatment, compared to control condition (Fig 4E and 4F). Of note, dexamethasone treated organoids reached adult enzyme activity levels at day 13 of culture, while control fetal organoids increased their enzyme activity values at a considerably slower rate. Dexamethasone did not increase *Treh* and *Arg2* gene expression levels (Fig 4C and 4D), but the activity level of the corresponding enzymes was significantly increased in dexamethasone treated fetal organoids at day 13 of culture compared to control condition (Fig 4G and 4H). Importantly, dexamethasone did not alter the enzyme activity of adult organoids (Appendix Figure S4). The effects of dexamethasone observed *in vitro* were similar to the effects *in vivo*, for example a precocious increase of sucrase-isomaltase mRNA levels after dexamethasone treatment and protective role on intestinal brush border by increased activites of Treh [31]. The accelerated suckling-to-weaning transition observed here demonstrates that the timing of the intrinsic intestinal maturation process observed in fetal organoids can be modulated by extrinsic factors.

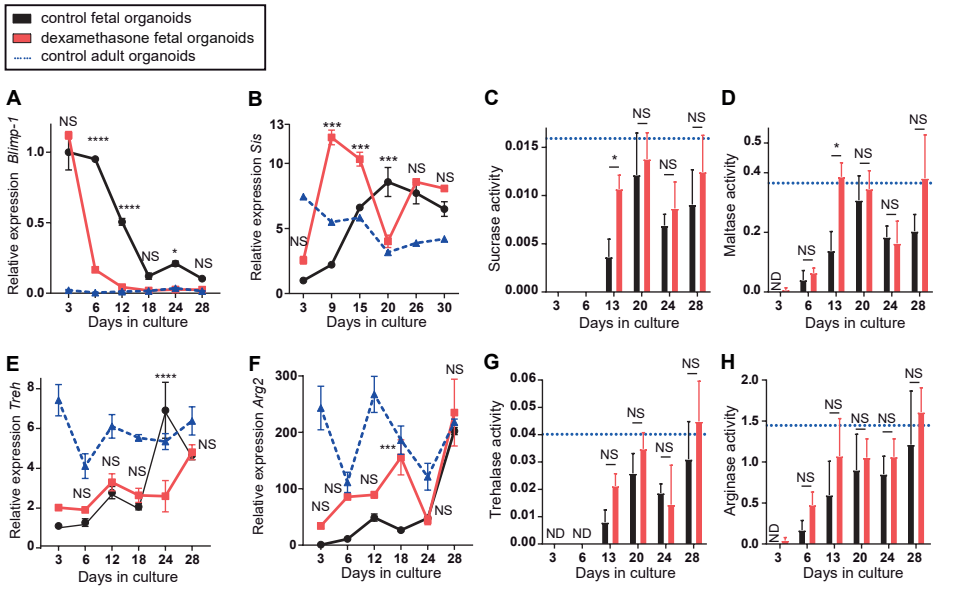


Figure 4 - **Dexamethasone accelerates maturation of fetal organoids.**

A, B, E, F Maturation is accelerated in dexamethasone treated fetal organoids ( ) compared to control ( ), as revealed by real-time qPCR. Decrease in relative expression of neonatal marker **A** *Blimp-1* and increase in expression of adult marker **B** *Sis* occur earlier in dexamethasone treated organoids, but expression of adult marker **E** *Treh* and **F** *Arg2* remains unchanged (n=3 individual wells from single organoid culture (see material and methods); experiment was repeated in four independent organoid cultures with similar results).

C,D,G,H Dexamethasone treatment accelerates increase in activity of **E** sucrase, **F** maltase, **G** trehalase and **H** arginase reaching adult organoid level ( ) at day 13 of the culture. Activity is given in µM glucose/µg protein·min^{-1} (n = 3 independent organoid cultures).

Data information: Data are presented as mean ± SEM. ND not detected, *p<0.05, ***p<0.001, NS not significant, between control and dexamethasone treated organoids (two-way ANOVA). Expression values of control fetal organoids and control adult organoids in B, E and F are the same as Figure 3E-G and enzyme activity levels of fetal organoids in C, D, G and H are the same as 3I-L.

Mixed organoid cultures: spheroids transit to organoids and do not reflect different maturation stages per se

Previously, it has been reported that organoid cultures are a mixed population of structures consisting of budding structures and hollow spheres, referred to as organoids and spheroids, respectively [32]. Furthermore, it has been shown that epithelial cells at E14-E16.5 propagate *in vitro* indefinitely as spheroids that do not transit to organoids even after continuous passaging [26,33]. In our E19 fetal cultures, we also observed two types of structures, organoids and spheroids (Fig 5A). Quantification of these two structures showed that at day 3 of *in vitro* culture, more than 80% of the outgrowths were spheroids, decreasing to approximately 10% at day 30 of culture (Fig 5B). The prevalence of spheroids at the start of the fetal cultures is in accordance with previous reports [26,33]. However, the count of spheroids decreased within the same passage (Fig EV5B), suggesting that spheroids transited to organoids within the same passage. In addition, after passaging we

typically observed a transient increase of spheroid number, albeit to a different degree, further suggesting interchangeable relationship between these two culture phenotypes (Fig EV5B). Such spheroid-organoids relationship has previously been described [32], suggesting that the spheroids that we observed are different from the ones generated from E14-E16.5 epithelium. In our culture conditions, most spheroids turned to budding organoids 2 days after each passage (Fig EV5E). Based on this observation, we consequently sampled at day 3 after each passage for all our analyses (Fig 1A). To determine the relationship between spheres and organoids, we separated spheroids and organoids from E19 fetal cultures at day 3 and cultured them independently in parallel with mixed culture (Fig EV5A). Counting of organoids and spheroids by equal vision field images of these three culture conditions (Figs EV5C-G) and live imaging of spheroid structures confirmed the interchangeable relationship between spheroids and organoids (Movies EV1 and 2). In conclusion, we observed seamless transition of spheroids to organoids within the same passage at early and late passaging. In addition, after each passage a proportion of organoids grew as spheroids though this seemed to decrease in time (Fig EV5).

Even though our experiments demonstrated transition of spheroids to organoids, intestinal maturation *in vitro* could still have been caused by a decrease in the number of spheroids during the 30 days of the culture period (see Fig EV5B). To further investigate this, expression of neonatal and adult markers was analyzed by immunohistochemistry (IHC) on mixed organoid cultures at different days/passages (Figs 5C-E). In early passages of mixed cultures, the neonatal marker Ass1 was expressed in both organoids and spheroids (Fig 5C), and, as expected, was virtually absent in late passages. In a sharp contrast, adult markers Sis and Arg2 were not present in spheroids neither in organoids of early cultures (day 6). However, these two enzymes were simultaneously expressed in spheroids as well as organoids at later culture time points (Figs 5D and E). These data suggest that spheroids, in our culture conditions, are appearing transiently, independently of the culture passage and maturation status of epithelial cells.

These data in conjunction indicate that fetal intestinal organoids cultured from late stages of fetal development (E19) to adulthood can be used as a novel *in vitro* model to study intestinal epithelial maturation, and open the way to decipher the impact of nutritional and environmental factors on this process reducing the need for (extensive) animal studies. In humans, there are multiple conditions in which gut maturation, i.e maturation of the epithelial digestive functions and gut barrier functions, is delayed or impaired. These conditions include e.g. preterm birth, undernutrition, and gastrointestinal infections with both short and long-term effects [34-37]. Short term effects may include necrotizing enterocolitis and sepsis in preterm infants and recurrent infections and environmental enteropathy in early life undernutrition. Long-term health conse-

quences may include stunting and neurodevelopmental impairment. Therefore, studies on gut maturation and how to modulate this process through early life nutrition are of pivotal importance for neonatal intestinal health and later life health.

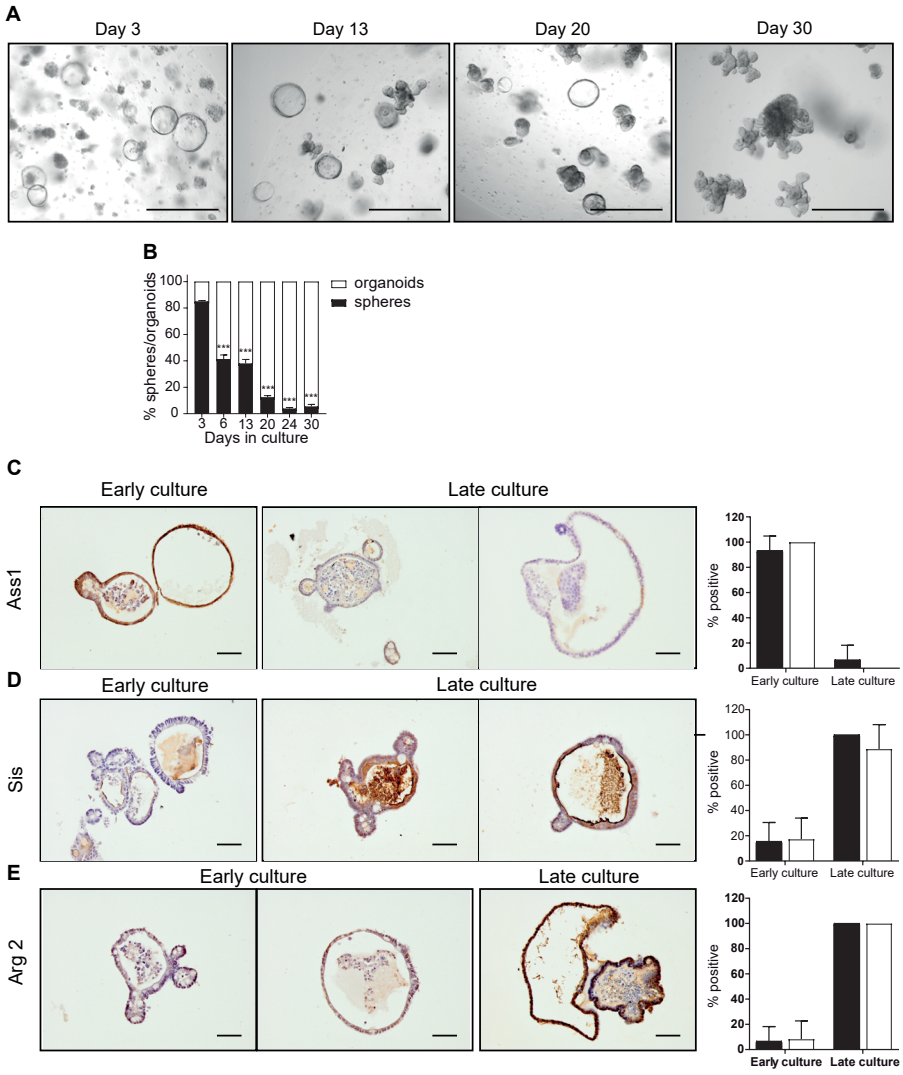


Figure 5 – **Spheroids and organoids show the same protein expression pattern, independently of culture stage/passage.**

A Microscopic images of fetal organoids at day 3, 13, 20 and 30 of culture. Scale bars: 500 µm.

B Percentage of fetal spheroids decreases during culture (n=3 individual wells from single organoid culture (see material and methods); experiment was repeated in ten independent organoid cultures with similar results).

C-E Immmunohistochemistry of fetal organoids for **C** Ass1 **D** Sis and **E** Arg2. Quantification of 3 slides per staining, 7 to 10 organoids / spheroids per slide. Scale bars: 50µm.

Data information: In B, data are presented as mean ± SEM. ***p<0.001, relative to number of spheroids at day 3 (one-way ANOVA).

Moreover, the development of cultured fetal intestinal organoids can be accelerated by dexamethasone as has also been described in *in vivo* studies, demonstating that cultured fetal intestinal epithelial cells can be used to identify novel factors that influence the timing of epithelial maturation. Such insights are fundamental for a better understanding of factors, e.g. bioactive components in human milk, prebiotics, probiotics and synbiotics that may promote gut maturation and thereby neonatal intestinal health in stressed conditions such as in preterm infants, undernourished infants, and infants with GI infections and inflammation.

EXPERIMENTAL PROCEDURES

Mice

Animal procedures complied with the guidelines of the EU and were approved by the Animal Welfare Body (ALC102556). Pregnant 8 weeks old C57Bl/6J mice were obtained from Jackson Laboratory and were sacrificed at day 19 of the pregnancy.

In vitro organoid culture

To generate single fetal organoid culture, fetuses from 2 mice were combined resulting in a final number of around 15 fetuses per experiment. For fetal and adult organoids culture, small intestine tissue was harvested, dissociated and cultured as previously described in a 48-well plate [38]. For adult organoids, the middle part of the small intestine of the mothers was used. In specific experiments, proximal and distal fetal intestinal tissue was separated based on location of stomach and appendix and cultured in parallel. Both fetal and adult organoids were maintained in ENR medium (Appendix Table S1) throughout the experiments. When mentioned, organoids were incubated with 0,01mM dexamethasone (Sigma-Aldrich) from day one of culture, continuously.

Separation of spheroids from organoids was performed at day 3 of culture, by collecting cultures in Cell Recovery solutions (Corning B.V.) and manually picking spheroids or organoids from suspension under microscope and separately re-culturing in Matrigel as described above.

Samples for RNA analyses, enzyme activity or immunohistochemistry were always taken 3 days after passaging of the culture. Counting was performed daily in the same wells, by 2 people, in 3 to 5 wells. Representative images of the cultures were taken by an inverted light microscope (Leica) from the same well, same field, on subsequent days of culture, within one passage. In addition, organoid cultures were monitored over time

by CytoSMART (Cytomate technologies B.V.) with a 30 minute snapshot interval, during several days of cultures. Images were processed to a movie using ImageJ.

RNA isolation and qRT-PCR

RNA was isolated using the Bioline ISOLATE II RNA Mini kit (BIO-52073, Bioline) according to manufacturers' instructions. RNA quality was measured on an Agilent 2100 Bioanalyzer, and only samples with a RNA integrity number (RIN) above 9 were included.

For transcriptome profiling, 400 ng RNA was amplified and labelled using 3' IVT pico kit Affymetrix RNA amplification kit (Nugene) according to manufacturer's protocol. Microarray analysis of mouse tissue and organoids was performed using Affymetrix Clariom® D 8-Array HT Plate according to the standard protocols of the Dutch Genomics Service and Support Provider (MAD, Science Park, University of Amsterdam, Netherlands). The data was normalised using Expression Console 1.4.1.46 and uploaded to R2: Genomics Analysis and Visualization Platform (http://hgserver1.amc.nl/). Microarray results were analysed using R2 software. Differentially expressed genes were selected based on fold change (≥ 2) in comparison to control group.

For qRT-PCR, 0,5 µg of RNA was transcribed using Revertaid reverse transcriptase according to protocol (Fermentas, Vilnius, Lithuania). Quantitative RT-PCR was performed on a BioRad iCycler using sensifast SYBR No-ROX Kit (GC-biotech Bio-98020) according to manufacturer's protocol. Cyclophilin was used as reference gene and relative gene expression was calculated using the $2^{-\text{delta Ct}}$ method. Primers sequences (specificity was tested using melting curve analyses) can be found in Appendix Table S2.

Enzyme activity

For the enzyme activity assay, organoids were washed with icecold PBS to remove matrigel, collected in cell lysis buffer (Cell Signalling Technology) and stored at -80°C until use. Lactase, sucrase, trehalase and maltase activity in the organoids were determined according to the method developed by Messer and Dahlqvist [39,40]. In short, samples were mild sonicated on ice for three seconds. For trehalase and maltase activity, samples were diluted five and ten times respectively. 30 µl of (diluted) organoid lysate was incubated with 30 µl of 0,12 M lactose (with p-chloromercuribenzoate as stabiliser to inhibit lysosomal p-galactosidase activity), 0,0112M maltose, 0,01M sucrose or 0,01M trehalose (all from Sigma-Aldrich) in 0,6M maleic-buffer (pH 6,0 Merck) for 60 minutes at 37°C. To determine the amount of glucose produced, 200 µl of the PGO-colour solution (10 U/mL glucose-oxidase from Aspergilus niger, 2 U/mL peroxidase and 8 mM o-dianisidine in 0,5M Tris-HCl buffer, pH 7,0; all from Sigma-Aldrich) was added and the absorbance was measured at 450 nm every five minutes for 30 minutes at 37°C. A glucose standard was

run in parallel to determine glucose production. Enzyme activity values were corrected for total amount of protein, as determined by BCA reaction[41], and are expressed as µM glucose/µg protein·min^{-1}.

Arginase activity was measured using the Arginase Activity Assay Kit (Sigma-Aldrich), according to the manufacturers' protocol. 40 µl of 5-times diluted organoid lysate was incubated with arginine in buffer (pH 9,5) supplemented with manganese, for two hours at 37°C. The urea produced was converted for one hour to a coloured product and absorbance was measured at 430 nm. An urea standard was run in parallel. Enzyme activity values were corrected for total protein, and are presented in units/L; one unit of arginase is the amount of enzyme that will convert 1,0 µmole of L-arginine to ornithine and urea per minute, at pH 9.5 and 37°C.

Immunohistochemistry

Tissue and organoids were fixed overnight in 4% formaldehyde, embedded in paraffin and sectioned. For staining, sections were deparaffinised with xylene and gradually rehydrated in ethanol. After blocking the endogenous peroxidase (0,01% H2O2 in methanol), slides were boiled for 20 min at 100 ° C on a heat block in 0,01M sodium citrate buffer (pH 6) for antigen retrieval. Slides were incubated overnight with primary antibody diluted in PBS with 1% bovine serum albumin and 0,1% Triton-X-100. Slides were washed with PBS and Powervision secondary antibody (Immunologic) was added for 30 minutes at room temperature, except for Blimp-1 staining that required 1h incubation with secondary antibody, followed by a 30 minute incubation with detection antibody. Antibody binding was visualized by adding chromagene substrate diaminobenzedine (Sigma-Aldrich) according to the manufacturer's protocol.

For whole-mount staining of organoids, organoids were collected from the matrigel by Cell Recovery Solution (Corning B.V.) and fixed overnight in 2% formaldehyde. After washing (PBS + glycine), permeabilization (PBS + 0.5% Triton X-100) and blocking (IF-wash + 10% goat serum), organoids were incubated with primary antibody for 1-2 hrs at RT. Staining was visualized with Alexa-conjugated secondary antibody (1 hr at RT) after which cells were mounted on a slide with ProLong™ Gold antifade reagent with DAPI (Invitrogen).

The following antibodies were used for immunohistochemistry: rabbit polyclonal anti-argininosuccinate synthethase I (1:10000, [42]), rat monoclonal anti-blimp1 (1:250, Santa Cruz, clone 6D3), rabbit polyclonal anti-rat IgG/biotinylated (1:200, Pierce Ab, 31834), streptavidin-HRP (K0675, DAKO), mouse monoclonal anti-lactase (1:4000, A. Quaroni, DRBB 2/33), rabbit polyclonal anti-mouse IgG/biotinylated (1:250, DAKO, ITK

A90-117B), rabbit polyclonal anti-arginase II (1:1000, [42]) and goat polyclonal anti-sucrase isomaltase (1:500, Santa Cruz, A17, sc27603). Antibodies used for immunofluorescence: rabbit polyclonal anti-mucin2 (1:500, Santa Cruz, sc-15334), rabbit polyclonal anti-lysozyme (1:500, DAKO, A0099), goat polyclonal anti-villin (1:100, Santa Cruz, sc-7672), rabbit polyclonal anti-synapthphysin (1:200, DAKO, A0010), goat anti-rabbit IgG/biotinylated (1:200, DAKO, E0432), streptavidin-FITC (1:250, DAKO, F0422), donkey anti-goat IgG-alexa647 (1:500, Invitrogen, A21447) and goat anti-rabbit IgG-alexa488 (1:500, Invitrogen, A11008).

Visualization of Lgr5 was performed using RNAscope®, an RNA in situ hybridization technique described previously [43]. RNAscope was performed according to the "Formalin-Fixed Paraffin-Embedded (FFPE) Sample Preparation and Pretreatment for RNAscope 2.5 assay" and "RNAscope 2.5 HD Detection Reagent – RED" protocols as provided by the manufacturer. For RNAscope the following probe was used: mm_Lgr5 (REF 312171, LOT 16250A).

Statistics

For all values, mean and standard error are given. One-way analysis of variance was used to test if an observed change over time was significant compared to day 3 of culture, with a Tukey post-test. To compare differences between two different conditions, two-way analysis of variance was performed with a Bonferroni post-test, unless indicated otherwise in the figure legend. *$p<0.05$, **$p<0.01$, ***$p<0.001$, NS = not significant.

Data Availability

The microarray data from this publication have been deposited to the GEO database (https://www.ncbi.nlm.nih.gov/geoprofiles/) and assigned the identifier GSE118982.

AUTHOR CONTRIBUTIONS

T.M.G. and M.N. designed and performed the experiments, analysed the data and wrote the manuscript. J.L.M.V., M.E.W., and S.M. contributed to experiments and data analysis. Project design and concepts were developed by I.B.R., R.M.v.E., G.R.vd.B. and V.M., who supervised the work and wrote the paper.

Acknowledgements

I.B.R. and R.M.v.E. are employees of Nutricia Research. G.R.vd.B. is employee of GlaxoSmithKline. This project was financially supported by Danone Nutricia Research.

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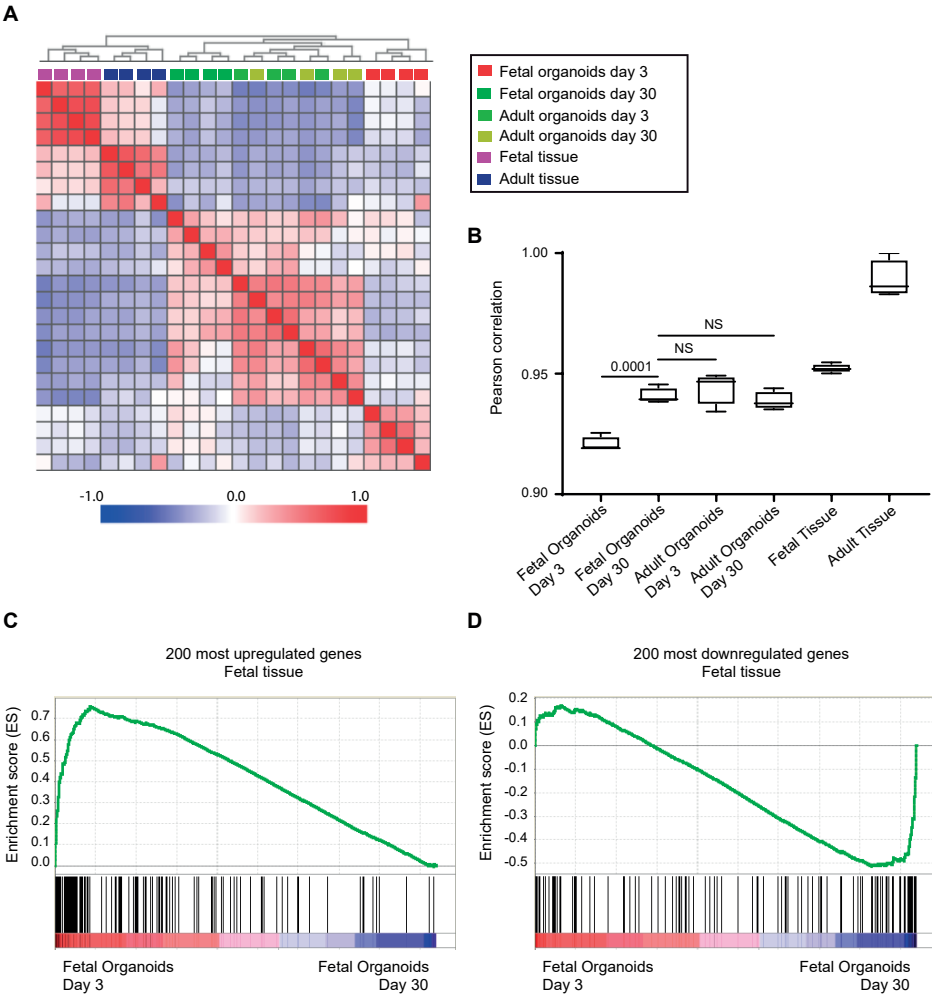

Figure EV1 – Comparison of organoid with tissue maturation.

A,B Pearson correlation analyses of fetal and adult organoids and fetal and adult tissue.

C,D Gene set enrichment analyses of 200 most **C** up and **D** down regulated genes from mouse primary fetal vs adult epithelium (GSE:35596) across fetal organoid maturation data set. Vertical lines below x-axis display relative distribution of expression per gene included in the geneset.

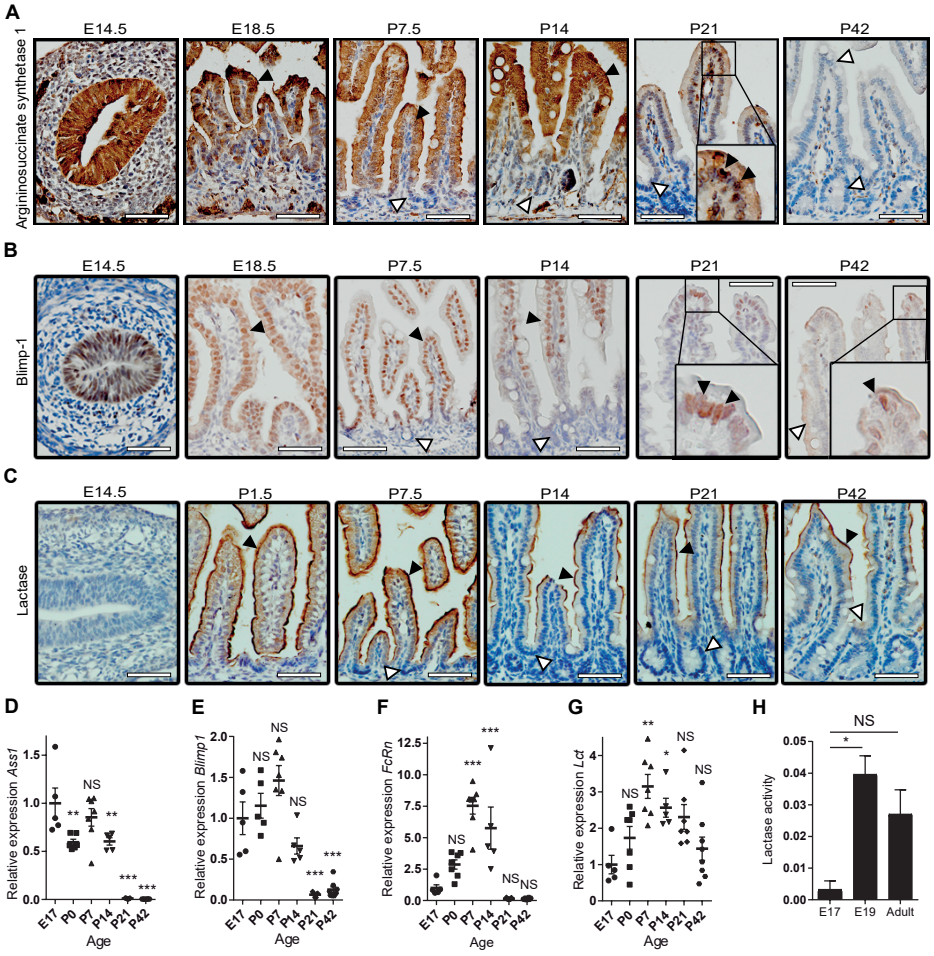

Figure EV2 – ***In vivo*** **expression of neonatal intestinal epithelial markers.**

A-C Immunohistochemistry of neonatal markers: **A** Ass1, **B** Blimp-1 and **C** Lct. Insets represent higher magnification of the rectangle. indicates negative cells and indicates positive cells. Scale bars: 50 µm.

D-G Whole tissue Real-time qPCR **D** Ass1, **E** Blimp-1, **F** FcRn and **G** Lct (n=5-8 individual intestinal specimens generated from offspring of single pregnant mice for E17 and P0-21, n=8 independent intestines of adult mice for P42).

H Enzyme activity assay of fetal and adult whole tissue lysates for lactase (n=3-5 individual intestinal specimens, generated from offspring of single pregnant mice for E17-19, n=5 independent intestines of adult mice for P42).

Data information: Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, NS not significant, in D-G relative to expression level at E17 (one-way ANOVA).

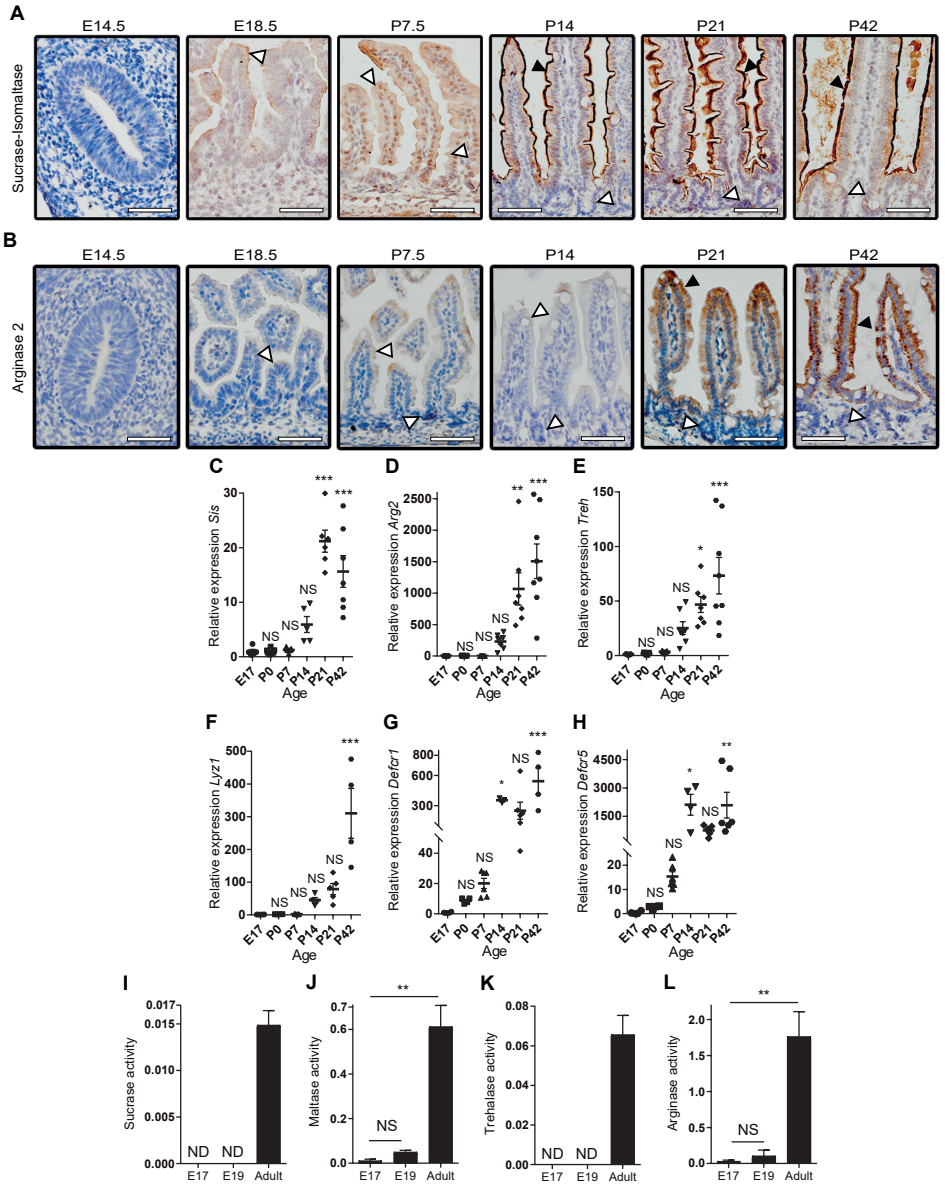

Figure EV3 – *In vivo* expression of adult intestinal epithelial markers.

A-B Immunohistochemistry of adult markers **A** Sis and **B** Arg2. Insets represent higher magnification of the rectangle. indicates negative cells and indicates positive cells. Scale bars: 50 µm.

C-H Whole tissue Real-time qPCR **C** Sis, **D** Arg2, **E** Treh, **F** Lyz1, **G** Defcr1 and **H** Defcr5 (n=5-8 individual intestinal specimens generated from offspring of single pregnant mice for E17 and P0-21, n=4-8 independent intestines of adult mice for P42).

I-L Enzyme activity assay of fetal and adult whole tissue lysates for **I** sucrase, **J** maltase, **K** trehalase and **L** arginase activities (n=3-5 individual intestinal specimens, generated from offspring of single pregnant mice for E17-19, n=5 independent intestines of adult mice for P42).

Data information: Data are presented as mean ± SEM. ND not detected, *p<0.05, **p<0.01, ***p<0.001, NS not significant, in C-H relative to expression level at E17 (one-way ANOVA).

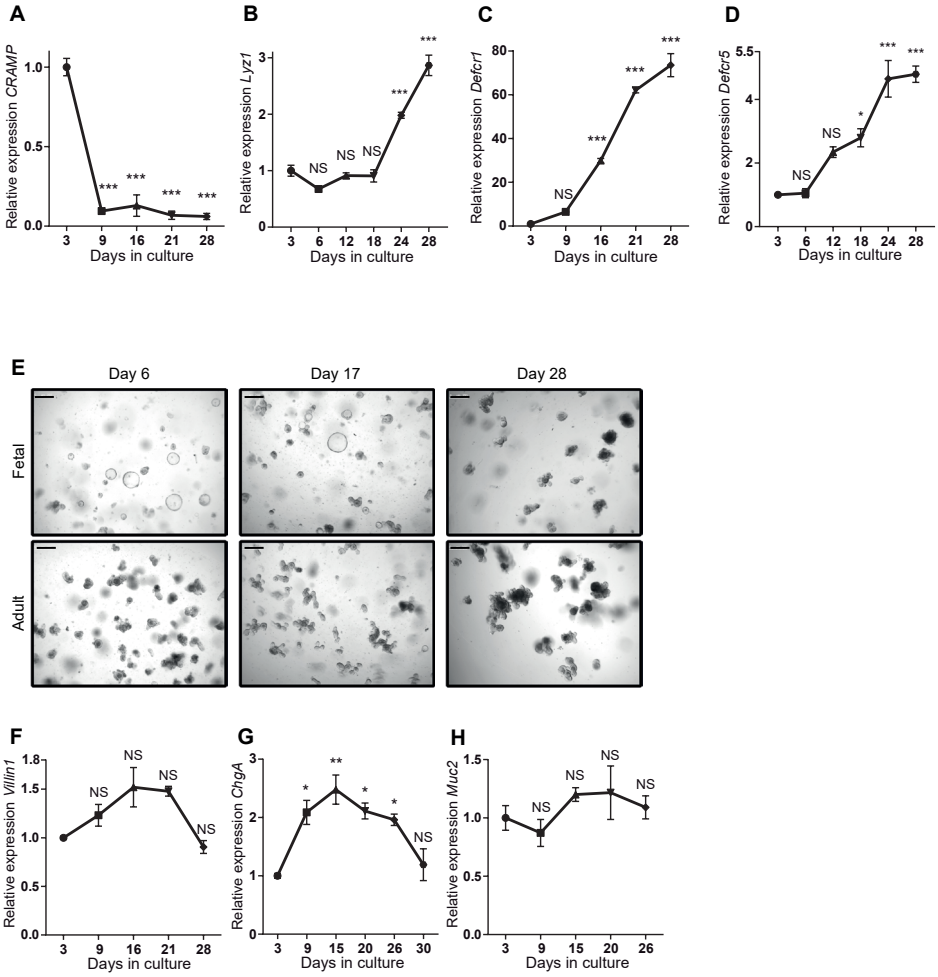

Figure EV4 - Fetal organoids mature *in vitro*.

A-D Real-time qPCR analysis of fetal organoids cultured for one month showing decrease in relative expression of **A** CRAMP and increase of the Paneth cell markers **B** Lyz1, **C** Defcr1 and **D** Defcr5. (n=3 individual wells from single organoid culture (see material and methods); experiment was repeated four times with similar results).

E Microscopic images of fetal and adult organoids at day 6, 17 and 28 of culture. Scale bars: 250 µm

F-H Real-time qPCR of **F** Villin1, **G** ChgA and **H** Muc2. (n=3 individual wells from single organoid culture (see material and methods); experiment was repeated four times with similar results).

Data information: Data are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, NS not significant, relative to expression level at day 3 (one-way ANOVA).

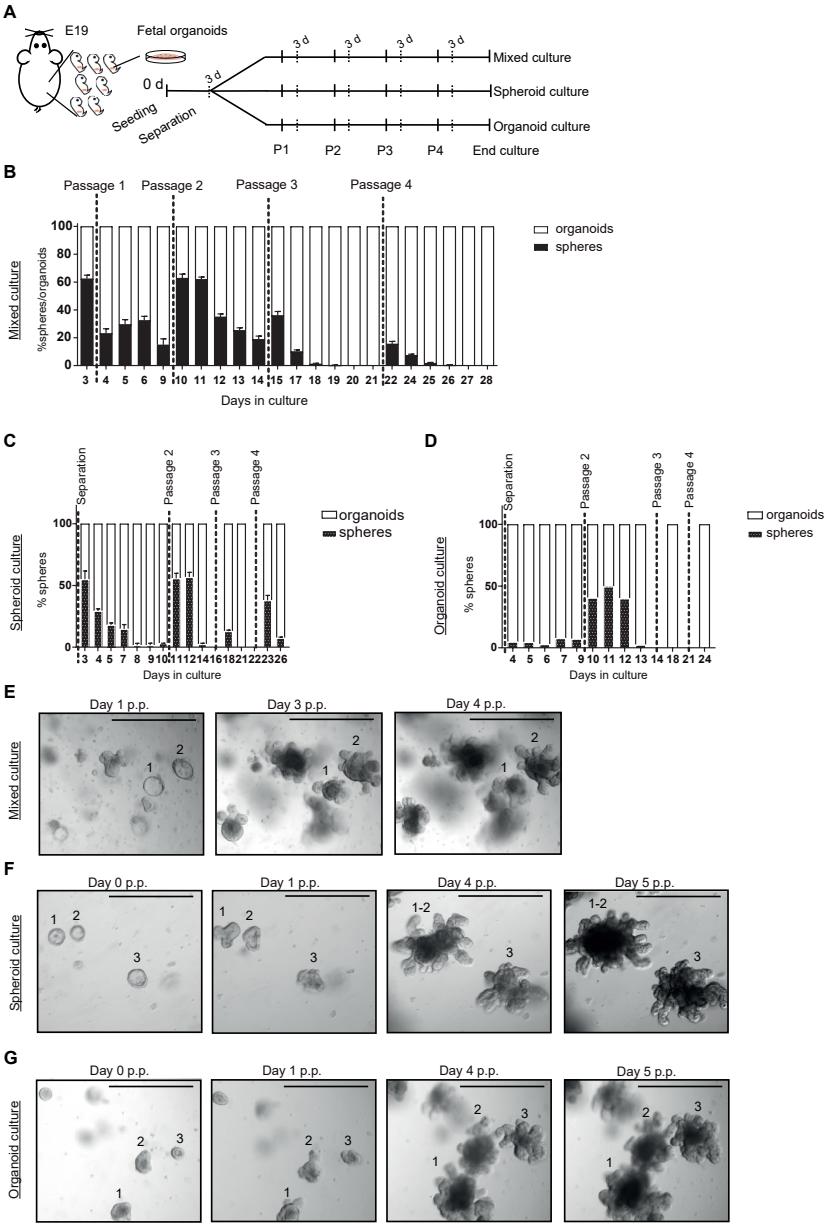

Figure EV5 – **Spheroid and organoid culture follow the same dynamics as mixed culture.**

A Scheme of culture separation.

B-D Percentage of spheroids vs organoids in **B** mixed culture, **C** spheroids and **D** organoids (n=3 individual wells from single organoid culture (see material and methods); experiment was repeated four times with similar results).

E Microscopic images of mixed culture at day 1, 3 and 4 post-passage (p.p.), showing the transition of spheroids 1 and 2 into organoids between passage 1 and passage 2. Scale bars: 500 µm.

F-G Microscopic images of **F** spheroid and **G** organoid culture at day 0, 1, 4 and 5 post-passage (p.p.), showing the conversion of spheroids 1, 2 and 3 into organoids between passage 1 and passage 2. Scale bars: 500 µm.

Data information: Data are presented as mean ± SEM.

3

Recapitulating Suckling-to-Weaning Transition In Vitro using Fetal Intestinal Organoids

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Journal of Visualized Experiments, 2019, 153: e60470

ABSTRACT

At the end of the suckling period, many mammalian species undergo major changes in the intestinal epithelium that are associated with the capability to digest solid food. This process is termed suckling-to-weaning transition and results in the replacement of neonatal epithelium with adult epithelium which goes hand in hand with metabolic and morphological adjustments. These complex developmental changes are the result of a genetic program that is intrinsic to the intestinal epithelial cells but can, to some extent, be modulated by extrinsic factors. Prolonged culture of mouse primary intestinal epithelial cells from late fetal period, recapitulates suckling-to-weaning transition in vitro. Here, we describe a detailed protocol for mouse fetal intestinal organoid culture best suited to model this process in vitro. We describe several useful assays designed to monitor the change of intestinal functions associated with suckling-to-weaning transition over time. Additionally, we include an example of an extrinsic factor that is capable to affect suckling-to-weaning transition in vivo, as a representation of modulating the timing of suckling-to-weaning transition in vitro. This in vitro approach can be used to study molecular mechanisms of the suckling-to-weaning transition as well as modulators of this process. Importantly, with respect to animal ethics in research, replacing *i*n vivo models by this in vitro model contributes to refinement of animal experiments and possibly to a reduction in the use of animals to study gut maturation processes.

INTRODUCTION

In many mammalian species, including mice and men, the neonatal intestine has several features that are distinct from the fully mature intestinal epithelium. These features facilitate neonatal enterocytes to digest and absorb milk, which contains high fat and low carbohydrates, with lactose as the major carbohydrate. The brush border of the neonatal intestinal epithelial cells express the disaccharidase lactase-phlorizin hydrolase (Lct)[1] to digest the milk disaccharide lactose. After the suckling period, enterocytes adapt to digest solid food that is rich in complex carbohydrates and low in fat. This is manifested by a switch in brush border disaccharidase expression from lactase to sucrase-isomaltase (Sis) and trehalase (Treh), which can digest more complex carbohydrates present in solid food[2]. Another metabolic switch is related to the low concentration of arginine in milk. To provide for the need for arginine, neonatal enterocytes express the rate limiting enzyme in arginine biosynthesis, argininosuccinate synthase-1 (Ass1), to synthetize arginine[3]. In contrast, adult enterocytes express arginase 2 (Arg2), an enzyme capable of catabolizing arginine that is abundant in solid foods. Furthermore, the neonatal intestinal epithelium expresses the neonatal Fc receptor for immunoglobulins (FcRn), which mediates absorption of the maternal IgG from the milk into the circulation/bloodstream[4]. The expression of FcRn declines significantly during the suckling-to-weaning transition[5]. In mice, maturation of Paneth cells occurs postnatally, coincidently with the formation and maturation of crypts, and is characterized by expression of antimicrobial peptides lysosome (Lyz) and defensins[6].

All these changes are part of the suckling-to-weaning transition, a process occurring gradually after birth to one month of age in mice, when the intestinal epithelium reaches its mature adult state. Suckling-to-weaning transition is intrinsically regulated and developmentally set in the gut tube. Transcription factor B lymphocyte-induced maturation protein-1 (Blimp-1) plays a key role in this intrinsic maturation process[7]. Blimp-1 is highly expressed in neonatal epithelium, while its expression decreases and is lost during the suckling-to-weaning transition and therefore can serve as a reliable marker of neonatal intestinal epithelium. Despite being an intrinsic process, the suckling-to-weaning transition can be modulated by external factors. For example, the synthetic analogue of cortisol, dexamethasone, is known to accelerate gut maturation in vivo[8,9].

Current in vitro models used to study intestinal epithelial maturation including the suckling-to-weaning transition, utilize adult epithelial cell lines and/or adult organoids which bear characteristics of adult intestinal epithelium. We have recently demonstrated that primary intestinal epithelial cells isolated from the late fetal intestine mature and recapitulate the suckling-to-weaning transition when growing in vitro as organoids[10].

We further showed that this gut maturation process in vitro occurs at the same pace as in vivo. Finally, we used dexamethasone to accelerate the maturation process in the same fashion described for *in vivo* studies.

Here, we outline a precise protocol for the isolation and culture of mouse late fetal intestinal organoids. We describe the preferred way of collecting samples for prolonged organoid culture and methods to monitor suckling-to-weaning transition in vitro. This protocol can be used for in vitro studies of intestinal epithelial maturation and modulators of this process and results in higher throughput, increased quality and translational value of the data and a reduction of animal use.

PROTOCOL

This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Ethic Committee for Animal Experimentation of the University of Amsterdam in full compliance to the national legislation on animal research following the European directive 2010/63/EU for the use of animals for scientific purposes (ALC312).

1. Isolation of fetal small intestinal organoids

1.1. Sacrifice the E18-E20 fetuses by decapitation with surgical scissors, according to the approved ethical regulations.

1.2. Immediately after euthanasia, carefully cut open the lower abdomen of the fetus with intestinal scissors and remove the whole intestine.

NOTE: Isolation must be performed with intestines between E18 and E20 of gestation in order to achieve the proper suckling-to-weaning transition and maturation in vitro.

1.3. With two small forceps, stretch the intestine (**Figure 1A**). Using the stomach and the appendix as guides, cut apart the proximal and distal part of the small intestine.

NOTE: If dissection is done carefully, it is possible to isolate the colon as well. Cut it apart using appendix as guide (**Figure 1B**).

1.4. Make the intestine open longitudinally: fix the intestine using a razor blade placed lengthwise and pull the intestine by sliding forceps (one arm of the forceps on

each side of the razor blade) along the razor blade. Subsequently, cut the opened intestine in 1 cm pieces.

1.5. Prepare two 50 mL tubes with 10 mL of ice cold phosphate-buffered saline (PBS). Transfer the proximal and distal part of the small intestine (and the colon if applicable) separately to the tubes. Keep the tubes on ice while dissecting the intestines of additional mice. Collect all intestinal parts of one litter together in the same tube.

NOTE: One litter has usually 6-10 fetuses. The intestines of all fetuses must be combined. This amount should be enough to yield 4-8 wells with organoids, in a 48-well plate, per intestinal part.

1.6. Proceed with organoid isolation as previously described[11]. In short, wash intestinal pieces with ice-cold PBS, incubate with 2 mM ethylenediaminetetraacetic acid (EDTA), dissociate the crypts from the tissue by harshly washing the pieces with ice cold PBS + 10% fetal calf serum (FCS), filter using a 70 µm cell strainer and centrifuge to collect the crypts at 150 x *g* for 5 min at 4 °C.

1.7. Plate 4 to 8 wells with crypts in extracellular matrix gel, depending on the pellet size, in a warm 48-well plate. Use 20 µL of crypts suspended in extracellular matrix gel per well. Let extracellular matrix gel solidify in a 37 °C incubator for 10 min.

NOTE: Considering that only 40% to 50% of isolated crypts form organoids, aim for a density of 250 to 300 organoids per well. First add less extracellular matrix gel than needed. Look under the microscope after platting the first well to analyze whether the density of the isolated crypts is ideal. If necessary, add more extracellular matrix gel.

1.8. In the meantime, prepare ENR medium: 14 mL of Advanced Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) 1:1 +++ (supplemented with 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 1x, L-glutamine 0.01 M and 0.2U/mL Penicillin/Streptomycin), 4 mL of Noggin-conditioned media, 2 mL of Rspondin-conditioned media, 400 µL of B27 supplement 1x, 200 µL of N2 supplement 1x, 50 µL of 1.25 mM n-Acetylcysteine, 20 µL of 0.05 µg/mL Epidermal Growth Factor (EGF).

NOTE: When culturing colon organoids, supplement with 50% Wnt conditioned media.

1.9. Add 250 µL of ENR medium per well.

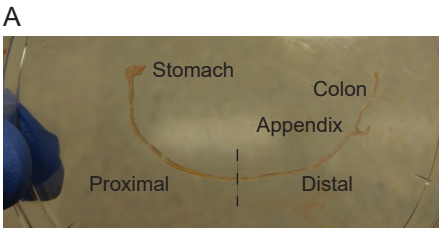

Figure 1: Isolation of mouse fetal small intestine.
(A) Photograph of dissected and stretched fetal gut, including stomach, proximal and distal small intestine, appendix and colon. Black line indicates where gut should be cut to divide the proximal and the distal small intestine.

2. Culturing of fetal organoids

2.1. Change medium of the cultures 3 times per week. Maturation of the organoids is achieved after 1 month of culture.

2.2. At day 3 after each passage, count the number of spheroids and organoids using an optical microscope. Quantify at least 3 wells per condition and all the organoids present in each well.

NOTE: Number of spheroids reduces with time, while the number of organoids increases (**Figure 2**).

2.3. Passage the organoids once a week by mechanical dissociation as described below.

2.3.1. Remove medium and add 1 mL of ice-cold Advanced DMEM/F12 1:1 +++. Collect all extracellular matrix gel with organoids into a 15 mL tube. Use a 200 µL tip on top of a 1000 µL filter tip and pipette up and down 20 times to disrupt the organoids.

2.3.2. Centrifuge at 150 x *g* for 5 min at 4 °C. Discard the supernatant and resuspend the pellet in 20 µL of extracellular matrix gel per well. Usually, fetal organoids can be expanded in a 1:2 ratio.

2.3.3. Let extracellular matrix gel solidify for 5-10 min. Add 250 µL of ENR medium per well.

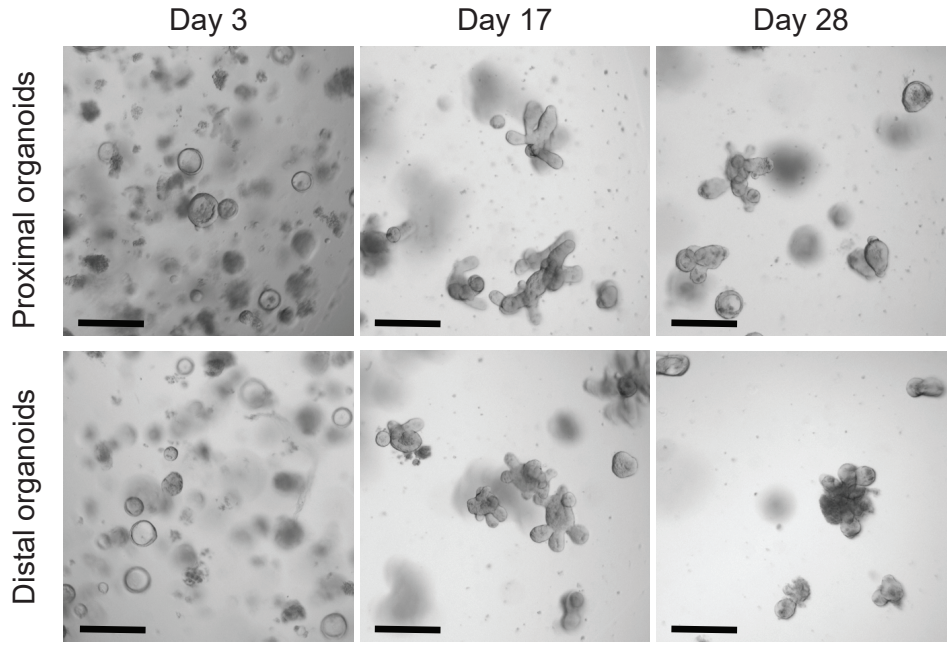

Figure 2: Representative microscopic images of proximal and distal fetal organoid culture at day 3, day 17 and day 28 of culture.
All images were obtained at day 3 after passage and show the decrease in the number of spheroids overtime. Scale bar: 500 µm.

3. Maturation analysis at RNA and protein level

3.1. Analyze culture every 3 days after each passage, for a period of 1 month (i.e., time in which complete maturation is achieved) (**Figure 3**).

NOTE: Fetal organoid culture is dynamic (Movie 1) and to avoid variation from the normal regeneration of organoids after mechanical disruption, it is necessary to always collect samples at the same day after passage.

3.2. RNA isolation

3.2.1. Collect 3 wells of organoids using 200 µL of RNA lysis buffer for each well supplemented with 2 µL of β-mercaptoethanol. After adding RNA lysis buffer+β-mercaptoethanol to the well, make sure all extracellular matrix gel with organoids is transferred to a RNase-free 1.5mL tube.

3.2.2. Vortex vigorously and keep at -80 °C for no longer than 1 month. Isolate RNA using commercially available silica spin column kits.

3.2.3. To increase RNA quality, after washing steps add 500 µL of 80% EtOH and gently mix by inverting the column. Centrifuge for 2 min at 11,000 x *g* to dry the column completely.

NOTE: Make sure to wash the inside of the lid of the tube with the 80% EtOH by flicking the tube upside down three to five times to get rid of all traces of guanidine thiocyanate.

3.2.4. To increase RNA yield, wait 1-2 min after applying RNase-free water before centrifuge. Re-elute RNA by applying the first flow-through eluate to the column a second time.

NOTE: Isolated RNA quality is sufficient for use in genome-wide expression analysis. Check whether RNA integrity number is above 8.

3.3. Protein isolation

3.3.1. Collect 5 wells of organoids using 250 µL of ice-cold cell recovery solution into a 15 mL tube. Incubate for at least 30 min on ice to dissolve the extracellular matrix gel (this will reduce protein contribution from extracellular matrix gel).

3.3.2. Wash with ice-cold PBS. Add 250 µL of cell lysis buffer and store at -80 °C.

NOTE: After sonication, samples can be used to detect enzyme activity or for Western Blots.

3.4. Immunostaining

3.4.1. Collect 2 wells of organoids using 250 µL of ice-cold cell recovery solution into a 15 mL tube. Incubate for at least 30 min on ice to dissolve the extracellular matrix gel (this will reduce staining background). Wash with ice-cold PBS.

3.4.2. Fix the organoids using 500 µL of of 4% paraformaldegyde (PFA) for 1 h at 4 °C. Wash with ice-cold PBS. Proceed to whole-organoid immunofluorescence or to paraffin embedding, according to published protocols[12].

NOTE: Use a plastic Pasteur pipette to handle the organoids. This will avoid disruption of its structure.

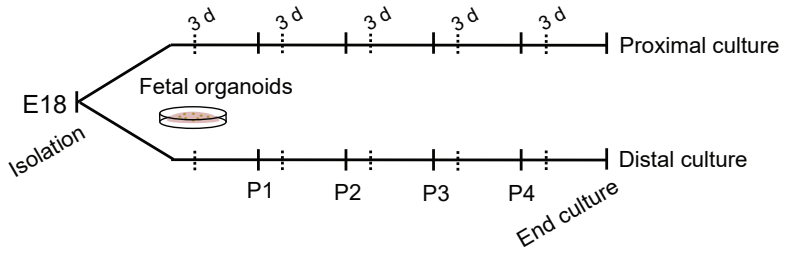

Figure 3: Schematic representation of organoid collection for analysis of gut maturation over time. Proximal and distal fetal organoid cultures should be cultured for one month and passaged every week. Samples for maturation analysis should be collected 3 days after isolation and every 3 days after each passage**.**

4. Effect of extrinsic factor (dexamethasone as an example) on organoid maturation process

4.1. On day one of culture, add 0.01M dexamethasone to the organoids. Incubate the organoids with dexamethasone during the whole month of culture by adding new dexamethasone every time medium is changed.

4.2. Gene expression analysis

4.2.1. Isolate RNA as described above. Synthesize, at the same time, cDNA of all samples to be compared (treated and untreated). Proceed with preferred qRTPCR method.

4.2.2. Use GeNorm to identify the two most stable reference gene every time a new treatment is used on the fetal organoids. Use the geometric mean of the two chosen reference genes for relative expression calculations.

NOTE: Suggestions of reference genes to test for mouse fetal organoids: Cyclophilin, Gapdh, βactin, 36b4, Hprt, Rpl4, Rpl32, Ppib and Tbp.

4.2.3. To investigate how a certain extrinsic factor affects postnatal mouse fetal maturation, all the following genes should be evaluated.

4.2.3.1. Check whether fetal markers lactase (*Lct*), argininosuccinate synthase 1 (*Ass1*), B lymphocyte-induced maturation protein 1 (*Blimp-1*) and neonatal Fc receptor (*FcRn*) decrease in expression during the first two weeks of culture and are absent for the remaining culture time (**Figure 4C**). Analyze whether this pattern is altered.

4.2.3.2. Check whether adult markers sucrase-isomaltase (*Sis*), arginase 2 (*Arg2*), trehalase (*Treh*) and lysozyme (*Lyz*) increase in expression after two weeks of culture (**Figure 4D**). Analyze whether this pattern is altered by the external factor.

NOTE: Dexamethasone is an external factor that can accelerate the maturation of the fetal organoids and can be used as a positive control in all experiments aimed to test other extrinsic factors.

4.3. Enzyme activity analysis

4.3.1. Isolate protein as described above. Detect intestinal disaccharidases activity according to protocols published by Dahlqvist and Messer[13,14].

4.3.2. Prepare 0.625 M maleic-buffer pH 6.0 (keep for 3 months at 4 °C). Using this buffer, prepare 0.05 M lactose (add p-hydroxymercuribenzoate sodium as stabilizer to inhibit lysosomal p-galactosidase activity); 0.05 M maltose; 0.05 M sucrose and 0.05 M trehalose.

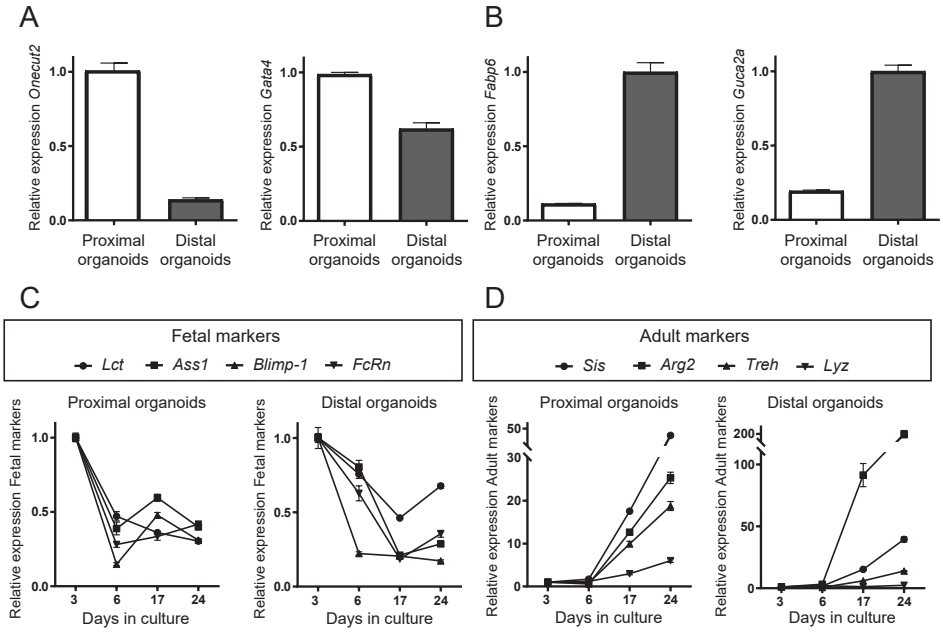

Figure 4: Representative qRTPCRs of gut maturation markers in proximal and distal fetal organoids. (**A**) Proximal markers *Onecut2* and *Gata4* are mainly expressed in the proximal organoid culture while (**B**) distal markers *Fabp6* and *Guca2a* are mostly expressed in the distal organoid culture. (**C**) Fetal markers *Lct*, *Ass1*, *Blimp-1* and *FcRn* decrease and (**D**) adult markers *Sis*, *Arg2, Treh* and *Lyz* increase over time in both proximal and distal organoid cultures. Error bars represent SEM.

NOTE: All these solutions can be kept for 5 days at 4 °C. Keep on ice while preparing assay.

4.3.3. Prepare assay standards by diluting 5.56 M glucose solution with ultrapure water to obtain solutions with the following concentrations: 0.125 M; 0.1 M; 0.075 M; 0.05 M and 0.025 M.

NOTE: solution stable for at least 3 months at 4 °C.

4.3.4. Incubate in a 96-well plate for 60 min at 37 °C:
- 30 µL of organoid lysate with 30µL of lactose
- 30 µL of organoid lysate with 30µL of sucrose
- 30 µL of ten times diluted organoid lysate with 30 µL of maltose
- 30 µL of five times diluted organoid lysate with 30 µL of trehalase
- 30 µL of undiluted organoid lysate with 30 µL of maleic acid, as sample background
- 30 µL of each glucose standard

- 30 µL of ultrapure water, as blank
- 30 µL of positive control (optimize dilution; lysed fetal intestinal tissue can be used as control for lactase activity while lysed adult intestinal tissue can be used as control for sucrase, maltase and trehalase)

NOTE: Dilution of samples should be made with cell lysis buffer. Keep the samples and plate on ice while preparing the assay.

4.3.5. To determine the amount of glucose produced by the enzymes present in the organoid lysate after incubation with their respective substrates, add quickly 200 µL of PGO-color solution and measure absorbance at 450 nm every 5 min for 30 min at 37 °C.

NOTE: Make PGO-color solution fresh. Use 10 U/mL glucose-oxidase, 2 U/mL peroxidase and 7.88 mmol/L o-dianisidine in 0.5mol/L Tris-HCl buffer at pH 7.0. Solution should be at room temperature when added to the plate.

4.3.6. Calculate activity according to glucose standard and correct for total amount of protein (determined by bicinchoninic acid assay (BCA)). Enzyme activity should be expressed as µM glucose/µg protein·min^{-1} (**Figure 5B**).

NOTE: Measure arginase activity using a commercially available arginase activity assay kit.

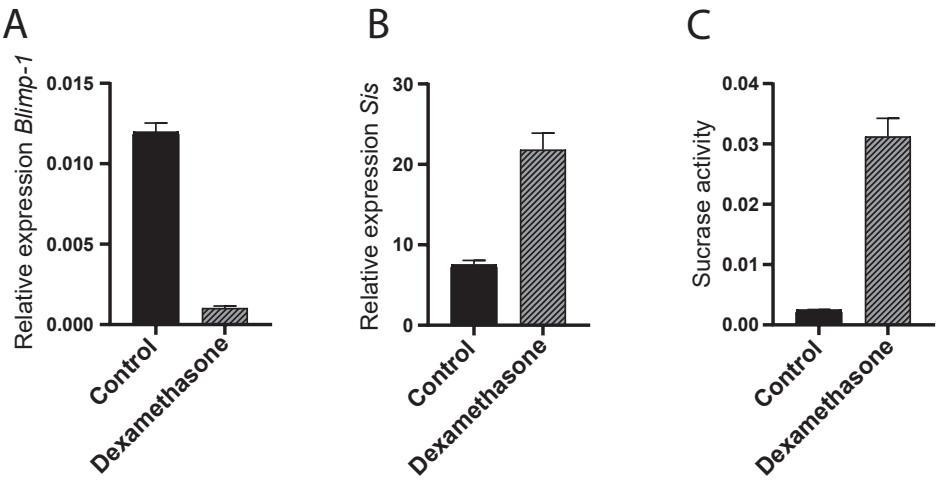

Figure 5: External factor dexamethasone can modulate the maturation of fetal organoids.
(**A**) Gene expression and enzyme activity of adult marker sucrase-isomaltase (Sis) is increased at day 12 of culture in dexamethasone treated organoids compared to control organoids, (**B**) while adult marker trehalase (Treh) is only increased at enzyme activity level. Error bars represent SEM.

DISCUSSION

This protocol describes culturing of late fetal intestinal organoids for prolonged time to mimic suckling-to-weaning transition in vitro. The process of maturation equals the pace in vivo and is completed after one month in culture. Downstream analysis of this culture using quantitative RNA and protein techniques are detailed.

In this protocol, primary intestinal cells from E18-E20 mouse embryos are used. The developmental stage of primary mouse cells used to generate organoids for this protocol is particularly important. Using earlier developmental stage will result in generation of intestinal spheroids that maintain their specific fetal gene expression over a prolonged period of time with limited transition to adult organoids[15,16]. Only late fetal stage spheroids are capable in transiting to adult organoids in vitro[10]. To maximize the window of opportunity with respect to impact of extrinsic factors on gut maturation, intestines from late fetal stage are recommended and not intestines from born pups that have been exposed to microbes and mother milk. It is reported that certain bacterial metabolites and milk components can act as modifiers of the maturation process[17].

To obtain sufficient amounts of cells to maintain the culture for one month to study the whole maturation from birth up to adulthood, while collecting the samples for downstream analyses, intestines from 6-8 embryos should be used as starting material. It is preferred to use embryos from the same developmental stage for generating the culture. We do not recommend pooling different litters as slight differences in developmental stage can influence expression of the maturation genes.

The protocol described here accounts for organoid generation from the proximal and distal small intestine to maintain developmental features of different segments of the gut. As an alternative, whole intestine can be used to investigate overall maturation with respect to the increase/decrease expression of the specific markers. In the latter case, fewer embryos could be used to isolate intestinal cells for starting culture.

This protocol is developed using three-dimensional organoid cultures. As organoids undergo dynamic growth in the culture, it is important to collect samples for downstream analyses at the same time point after passaging. In this protocol, we have selected day 3 after passage, as it represents the medium time between two splits at which organoids contain robust buds and little to no cell death. An alternate time point after passaging can be used, but it should be consistent during the whole experiment. We do not recommend growing organoids for more than 7 days after a passage, as increase of death cells in the organoid lumen can affect the results.

In our experiments, we have used dexamethasone as an example of an extrinsic factor that is shown and best studied in literature to accelerate intestinal maturation in vivo[9,18]. Dexamethasone exerts its effects via both genomic and non-genomic routes. For example, on the level of genomic regulation, a precocious increase of *Sis* mRNA levels can be observed. On a non-genomic level, we observe alterations in the activity of digestive enzymes such as trehalase. Both are in accordance with described specific aspects of dexamethasone on sucrase gene activation and non-genomic activating effect on intestinal brush border enzymes observed in vivo[19]. The fact that extrinsic factors, like synthetic glucocorticoids, can modulate certain aspects of suckling-to-weaning transition in the organoid culture, similarly to that described in vivo, further establishes the mouse fetal intestinal organoids as a model for the investigation of different kind of modulators of gut maturation.

Even though the morphological maturation of human intestinal epithelium is completed in utero at gestational stage of 22 weeks, the intestinal barrier function matures till childhood in a close relationship with the type of feeding, development of microbiota and immune response. Due to the limited availability of human tissues at these developmental stages, the translational value of in vitro murine model lies in the possibility of high throughput screens of factors capable of modulating intestinal maturation, a process conserved among suckling mammals.

Importantly, with respect to animal ethics in research, this model can contribute to refinement of animal experiments as it does not include interventions performed on animals. The number of animals can be further reduced by redesigning research questions to one or two time points of culture which will allow testing of multiple components within one culture.

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4

Early Life Antibiotics Influence In Vivo And In Vitro Mouse Intestinal Epithelium Maturation And Functioning

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Cellular and Molecular Gastroenterology and Hepatology, 2021, 12(3): 943-981

ABSTRACT

Background & Aims

The use of antibiotics (AB) is a common practice during the first months of life. AB can perturb the intestinal microbiota, indirectly influencing the intestinal epithelial cells (IECs), but also directly affect IECs, independent of the microbiota. Previous studies have mostly focused on the impact of AB treatment during adulthood. However, the difference between the adult and neonatal intestine warrants careful investigation of the AB effects in early life.

Methods

Neonatal mice were treated with a combination of amoxicillin, vancomycin, and metronidazole, from postnatal day 10 to 20. Intestinal permeability and whole intestine gene and protein expression were analyzed. IECs were FACS-sorted and their genome-wide gene expression analyzed. Mouse fetal intestinal organoids were treated with the same AB combination and their gene and protein expression, and metabolic capacity determined.

Results

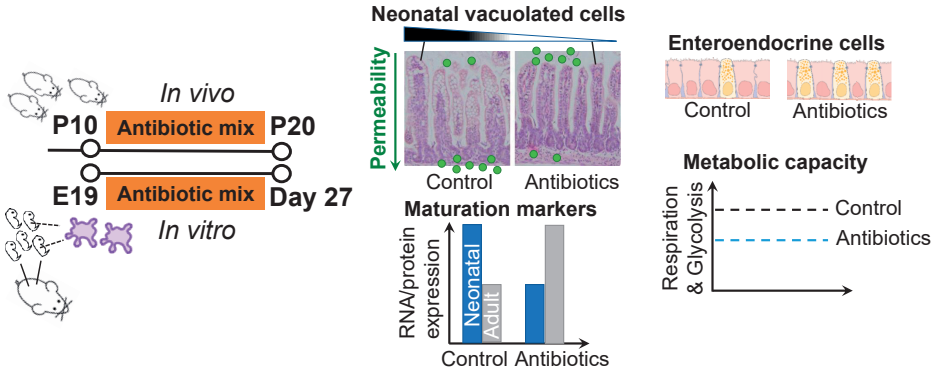
We found that *in vivo* treatment of neonatal mice led to decreased intestinal permeability and reduced number of specialized vacuolated cells, characteristic of the neonatal period and necessary for absorption of milk macromolecules. Additionally, the expression of genes typically present in the neonatal intestinal epithelium was lower, whereas the adult gene expression signature was higher. Moreover, we found altered epithelial defense and transepithelial sensing capacity. *In vitro* treatment of intestinal fetal organoids with AB showed that part of the consequences observed *in vivo* is a result of a direct action of the AB on IECs. Lastly, AB reduced the metabolic capacity of intestinal fetal organoids.

Conclusion

Our results demonstrate that early life AB treatment induces direct and indirect effects on IECs, influencing their maturation and functioning.

KEYWORDS

Antibiotic treatment; Neonatal intestine; Fetal organoids


In vivo
P10
Antibiotic mix
P20
E19
Antibiotic mix
Day 27
In vitro
Neonatal vacuolated cells
Permeability
Control
Antibiotics
Maturation markers
RNA/protein expression
Neonatal
Adult
Control
Antibiotics
Enteroendocrine cells
Control
Antibiotics
Metabolic capacity
Respiration & Glycolysis
Control
Antibiotics

INTRODUCTION

Infectious diseases are one of the leading causes of mortality in children under the age of 5 years. Since antibiotics (AB) are the cornerstone of adequate treatment of bacterial infections, it is not surprising that AB are the most prescribed drugs in early childhood [1-3]. Often, AB of different classes are used in combination in order to treat suspected or confirmed infections caused by different pathogens. Three frequently used AB in children are amoxicillin, metronidazole, and vancomycin [1, 3-11]. Amoxicillin is prescribed when there is suspicion of systemic infection, affecting both gram-positive and gram-negative bacteria [8, 12-14]. When gastrointestinal complications develop, caused by anaerobic bacteria, metronidazole is given, frequently in combination with amoxicillin [11, 15-18] Upon onset of systemic or gastrointestinal infection with gram-positive bacteria, especially in the case of *Staphylococcus aureus* and *Clostridium difficile*, vancomycin is administered [5, 8, 11, 19-22]. The combined use of the above-mentioned AB is as well common [8, 11, 17, 20-22]. Although AB have a crucial and beneficial role in treating bacterial infections, they also have several short and long-term detrimental effects. Use of AB in early life is associated with necrotizing enterocolitis (NEC) [23-25], infantile colics [26, 27], and eczema. Later in life, diseases such as allergy [28-30], obesity [31-34], and inflammatory bowel diseases (IBD) [35-38] have also been linked with prolonged AB exposure early in life. Yet, how AB can affect host cells and therefore contribute to disease development is still not clear.

Increased susceptibility to diseases after AB treatment can be a result of both indirect and direct effects on host cells. AB disturb the microbial community which indirectly affects gut homeostasis and perturbs the function of intestinal epithelial cells (IECs) [35, 39-47]. When microbiota disturbance occurs during a specific neonatal time window, the development of the gut immune system is compromised, leaving the organism more sensitive to immune pathologies later in life [30, 48-54]. At the same time, AB can directly influence IECs, independent of the microbiota [55-57]. It has been shown that AB directly elicit various immunomodulatory effects [58]. Furthermore, it was found that prolonged treatment of human fibroblasts with specific antibiotics affects mitochondrial respiration [59]. More recently, it was shown that one-third of the AB-induced changes in host intestinal epithelial gene expression could be attributed to direct regulation of their expression by the AB, and not by a shift to a different microbiota composition [57].

In previous studies, the impact of AB on the intestine and the intestinal epithelium was investigated in adult mice [39-41, 44-46, 57]. However, the effects of AB in adult mice are likely not the same as in neonatal mice, as the gut of neonatal mice has not gained its full function and is still maturing up until weaning, including the suckling-to-weaning

transition [60-62]. Intestinal maturation *in vivo* has been proposed to occur in a wave from proximal to distal intestinal regions [63]. In mice, this process takes place from postnatal day (P) 14 to P28 and prepares the intestine for the switch to a solid diet, resulting in several changes in epithelial cell functions [61]. The immature intestinal epithelium is characterized by the presence of an apical canalicular system (ACS) on the apical side of the absorptive enterocytes [64-67]. ACSs are responsible for the active endocytosis of maternal immunoglobulins and macromolecules present in milk and feed into supranuclear vacuoles, which are most abundant in the distal small intestine [68, 69]. The presence of ACSs contributes to the increased intestinal permeability that is characteristic of the neonatal period and gradually decreases due to the replacement of cells containing ACSs with adult enterocytes [68, 70]. Other markers of the immature intestine are the neonatal Fc receptor (FcRn), which mediates the uptake of IgG from breast milk [71, 72], and argininosuccinate synthase 1 (Ass1), the rate-limiting enzyme in the arginine biosynthesis a semi-essential amino acid that is not present in milk [73]. During the suckling-to-weaning transition, vacuolated enterocytes disappear, the intestinal epithelium tightens and its permeability becomes more selective [72]. The mentioned neonatal markers decrease in expression, while adult brush-border enzymes sucrase-isomaltase (Sis) [74] and arginase 2 (Arg2) [75] start to be expressed to digest solid food. The emerging Paneth cells produce Lysozyme-1 (Lyz1) and Reg-3 lectins [76-79] and by one month of age, the intestine is fully matured and has achieved all adult characteristics. Recently, our group developed an *in vitro* model to study gut neonatal development utilizing a prolonged culture of fetal mice gut organoids. We established that fetal organoids isolated from the intestine of mice at late fetal stage (embryonic day E19) undergo intrinsic maturation *in vitro,* recapitulate suckling-to-weaning transition, and that extrinsic factors can be applied to the culture to investigate whether they can modulate intestinal epithelial maturation [80, 81].

Here, we combined two different approaches to study how AB affect the neonatal intestinal epithelium. First, we investigated the effect of early life AB on neonatal IECs in mice, either due to indirect or direct mechanisms and second, we determined the direct effect of AB on IECs in organoids.

RESULTS

Mice pups treated with early life antibiotics show decreased intestinal permeability and loss of vacuolated enterocytes

To investigate the effects of AB during neonatal development *in vivo*, we treated mice with AB as of postnatal day 10 (P10) since this time point of murine intestinal develop-

ment corresponds to the newborn human intestine [82]. P10 mice were daily and orally treated for 10 days with the combination of three frequently used AB in neonates and children: amoxicillin (β-lactam), vancomycin (glycopeptide), and metronidazole (nitroimidazole) [1, 3, 5, 6, 8, 10, 83] (Fig. 1A). During this period, weight gain was similar in AB-treated and control pups (Fig. 1B), indicating that the AB treatment did not affect growth. At postnatal day 20 (P20), the small intestine was significantly heavier in the AB-treated pups compared to control pups, though the intestinal length was similar in both groups (Fig. 1C and 1D). The liver weight was similar between both groups, but spleens of AB-treated pups were significantly heavier, which could indicate an inflammatory process (Fig. 1E and 1F).

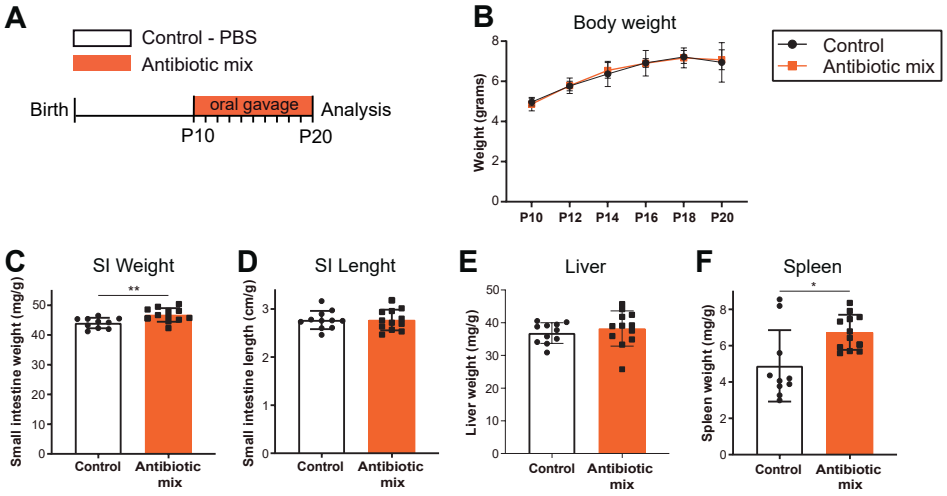


Figure 1. *In vivo* growth and macroscopic assessment of small intestine, liver, and spleen.
(**A**) Experimental design of *in vivo* antibiotic treatment of pups between postnatal (P) day 10 and P20. Antibiotic mix: amoxicillin, metronidazole, and vancomycin. All analyses were done at P20. (**B**) Weight of pups was measured every two days during antibiotic mix treatment between P10 and P20. (**C**) Small intestine weight, relative to body weight. (**D**) Small intestine length, relative to body weight. (**E**) Liver weight, relative to body weight. (**F**) Spleen weight, relative to body weight. Statistical analysis was performed by two-way ANOVA test with Sidak's multiple comparisons test (**B**) or two-tailed unpaired t-test (**C-F**). Error bars indicate mean ± SD. Levels of significance are indicated (*p<0.05, **p<0.01). *n* = 9-12 pups per group.

As the small intestine (SI) follows a maturation wave along the proximal-to-distal axis [63], which displays distinct functional and genetic profiles [84-86] that can be differently affected by AB treatment [87], we analyzed the proximal and distal parts of the SI separately. The histology of the SI revealed no major differences in overall morphology (Fig. 2A). However, we observed a strong reduction in the number of vacuolated enterocytes containing ACSs in the distal SI after AB treatment (Fig. 2A and 2B). Intestinal permeability as measured by the passage of fluorescein isothiocyanate (FITC) dextran through the intestinal epithelial barrier, was significantly lower in AB-treated pups com-

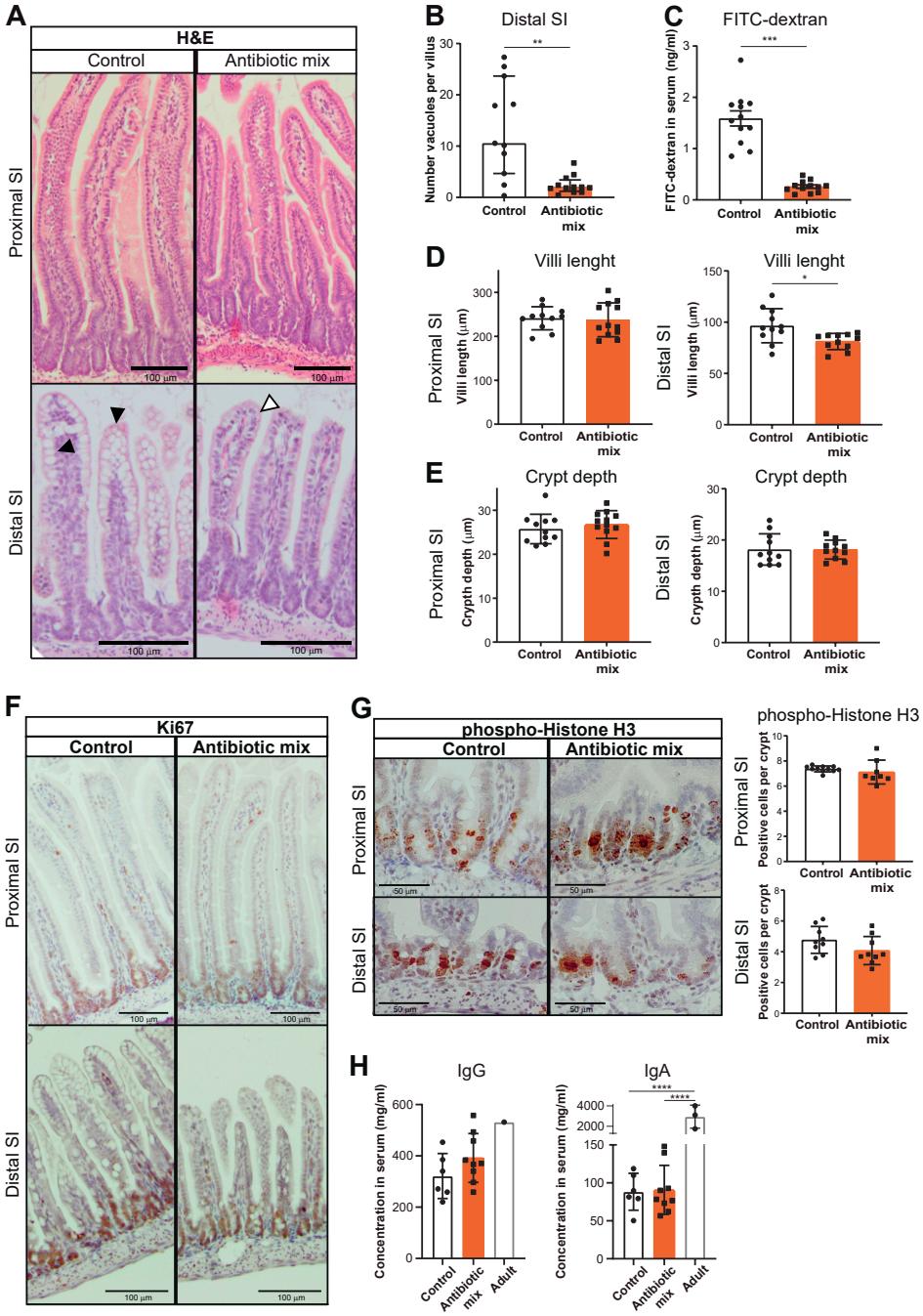


Figure 2. Early life antibiotics affect intestinal barrier function *in vivo* particularly in the distal small intestine.

(continued) Figure 2. Early life antibiotics affect intestinal barrier function *in vivo* particularly in the distal small intestine.

(**A**) H&E staining of proximal and distal small intestine. Black triangles indicate vacuolated enterocytes and white triangles indicate non-vacuolated enterocytes. (Scale bars, 100 µm.) (**B**) Quantification of the number of vacuoles per villi in distal small intestine. (**C**) Permeability assay assessed by FITC-dextran concentration in serum 4 hours after oral gavage. (**D**) Villi length in proximal and distal small intestine. (**E**) Crypt depth in proximal and distal small intestine. (**F** and **G**) Immunohistochemistry of proliferation markers Ki67 (**F**) and phosphorylated histone H3 (**G**) in proximal and distal small intestine. (Scale bars, 100 µm.) (**H**) Serum concentration of immunoglobulin G (IgG) and immunoglobulin A (IgA) in control and antibiotic mix treated pups at P20 compared to adult mice.

Statistical analysis was performed by Mann-Whitney test as data was not normally distributed when assessed by D'Agostino and Pearson normality test (**B**) or two-tailed unpaired t-test (**C-E** and **G**) or one-way ANOVA with Tukey's multiple comparisons test (**H**). Error bars indicate median with interquartile range (**B**) or mean ± SEM (**C-E** and **G-H**). Levels of significance are indicated ((*p<0.05,**p<0.01, ***p<0.0001, ****p<0.0001). *n* = 9-12 pups per group (**B-E** and **G**) and *n* = 6-9 P20 samples per group and *n* = 1-3 adult samples (**H**).

pared to control pups (Fig. 2C). While the length of the villi was similar in the proximal SI, villi in the distal SI were shorter in AB-treated pups compared to control pups (Fig. 2D). Yet, crypt depth in proximal and distal SI was not affected by AB treatment (Fig. 2E). Cellular proliferation was similar between the two groups, in both proximal and distal SI, as shown by immunodetection of the proliferation marker Ki67 (Fig. 2F), though the mitotic marker phosphorylated histone H3 followed a trend towards a lower number of proliferating cells in the distal SI (Fig. 2G). Since we observed a strong reduction in both ACS and intestinal permeability, we examined whether AB exposure decreased the transfer of immunoglobulins through the neonatal intestinal epithelium. Serum IgG and IgA concentrations as measured by ELISA assay were similar between the two groups at P20 (Fig. 2H). The decreased number of vacuolated enterocytes and reduced intestinal permeability suggest an effect of early life AB on the maturation of the intestinal barrier.

Early life antibiotics induce gene expression changes in small intestine epithelial cells *in vivo*

Decreased intestinal barrier and disappearance of ACS are the prime characteristics of the intestinal maturation process occurring during the replacement of neonatal epithelium by adult epithelium. To determine the effect of the AB treatment on intestinal epithelial maturation, we performed genome-wide gene expression analysis on mRNA from proximal and distal SI epithelial cells (Fig. 3A). The epithelial marker EpCAM was used to isolate the epithelial cells of both regions, as no difference in the percentage of this marker was detected between control and antibiotic-treated samples (Fig. 3B). Principal component analysis (PCA) showed a clear separation between proximal and distal SI epithelial cells along with the first component (PC1 44.3%) (Fig. 3C). Despite some variance between samples, AB-treated epithelial cells separated from control epithelial cells along PC2 (PC2 13.2%). This separation between AB and control groups was more evident in the epithelial cells of the distal SI compared to the proximal SI (Fig. 3C). Indeed, the differential gene expression analysis revealed 67 genes in proximal SI

and 634 genes in distal SI epithelial cells that were upregulated or downregulated 2-fold or more in the samples treated with early life AB (Fig. 3D, Table S1 and S2).

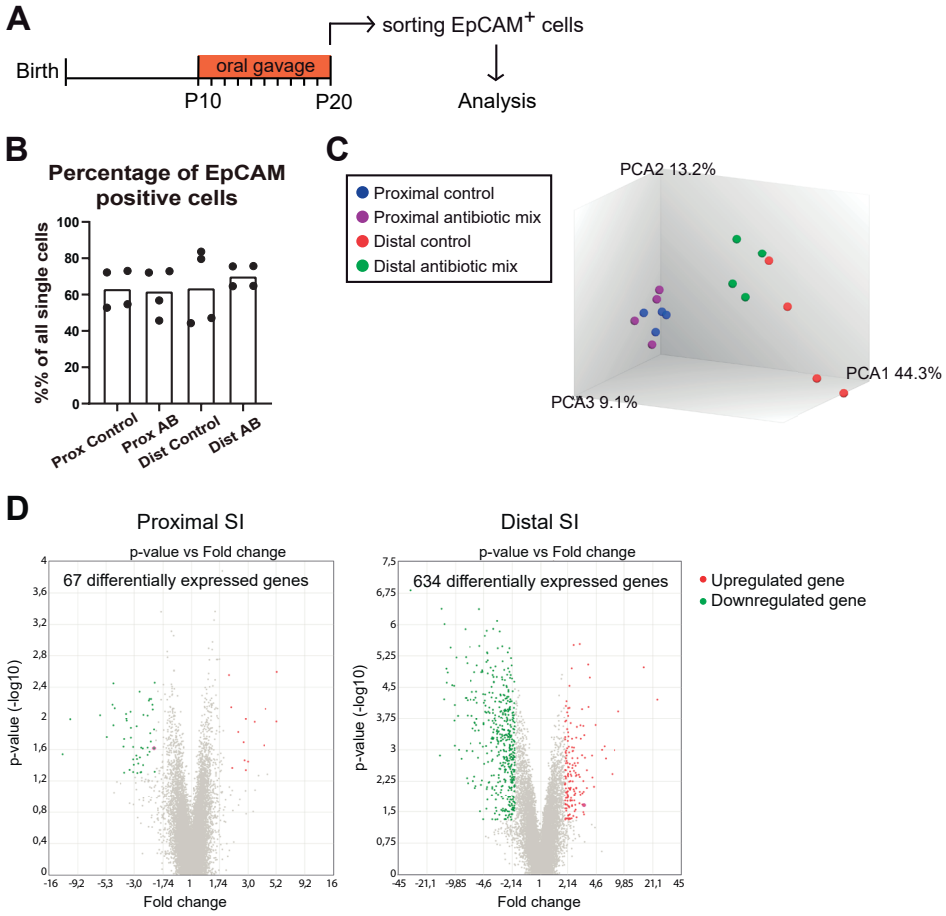


Figure 3. Genome-wide gene expression analysis of sorted intestinal epithelial cells.
(**A**) Experimental design of genome-wide gene expression analysis of FACS-sorted P20 intestinal epithelial cells. (**B**) Percentage of EpCAM stained cells in the total number of FACS-sorted intestinal epithelial cells per condition. (**C**) PCA analysis of sorted epithelial cells from P20 proximal and distal small intestine treated with the antibiotic mix or PBS (control). (**D**) Volcano plots of microarray analysis showing genes differentially expressed between control and antibiotic-treated FACS-sorted P20 intestinal epithelial cells. Green dots identify downregulated genes and red dots identify upregulated genes. Statistical analysis by ANOVA eBayes, $p < 0.05$ cut-off. $n = 4$ samples per group.

Within the sets of the most differentially expressed genes, many of the known fetal/neonatal and adult maturation markers were changed in the distal SI (Fig. 4A). Specifically, fetal/neonatal markers *FcRn*, *Blimp-1, Ass1,* and *Slc43a3* were downregulated after AB treatment and adult markers *Sis*, *Arg2, Dpep1*, *Pmp22*, *Gjb3,* and *Slc13a1* were upregulated in epithelial cells of AB-treated pups (Fig. 4A). These differences were less obvious in the proximal SI (Fig. 4A). In addition, in the distal SI, intracellular digestion genes, i.e.

genes involved in the formation of the lytic supranuclear vacuoles that compose the ACS (*Slc46a3I*, *Tmem9*, *Dab2*, *Mcoln3*, *Glmp*) as well as genes related to the degradation of milk macromolecules within these vacuoles (*Hyal5*, several *Cts*'s, *Galns*, *Neu1*, *Lgmn*, *Lipa*, *Man2b2*), were significantly downregulated in AB-treated pups (Fig. 4B). The differences in intracellular digestion genes were less evident in proximal SI epithelial cells (Fig. 4B). Accordingly, gene set enrichment analysis (GSEA) using sets of genes from the Molecular Signatures Database (MSigDB), such as HALLMARK and Gene Ontology (GO), showed "HALLMARK Vacuole organization" and "GO Fatty acid metabolism" gene sets enriched in distal SI epithelial cells isolated from control pups (Fig. 4C and 4D). Furthermore, changes in (innate) defense genes were also observed in the distal SI after AB treatment, such as upregulation of lysozyme-1 (*Lyz1*) and Reg-3 lectins (Fig. 4E). AB-treated pups also showed lipid, short-chain fatty acids (SCFA), and bile acid (BA) metabolism in distal SI epithelial cells being affected by early life AB: lipid transporter *Apoa4*, cholesterol transporters *Abcg8* and *Npc1l1*, lipid metabolism enzymes *Acot5*, *Acot12*, *Acadl, Acsl1* and *Fabp6,* and SCFA mitochondrial enzyme *Acss1* were downregulated and primary BA uptake transporter *AsbtI*/*Slc10a2* was upregulated (Table S2). Moreover, distal SI epithelial cells of pups treated with AB revealed downregulation of genes encoding subunits of the mitochondrial complex IV (*Cox6b2*) and complex V (*Atp5e*), as well as downregulation of *Slc2a2*/*Glut2* and *Slc37a4*, and upregulation of *Slc2a1/Glut1*, all glucose transporters (Table S2).

Of the differentially expressed genes in the proximal SI epithelial cells, the top upregulated genes were markers of enteroendocrine cells (Fig. 4F). This secretory cell type functions as (trans)epithelial sensors within the gut in order to regulate energy homeostasis by producing hormones, like neurotensin (*Nts*), somatostatin (*Sst*), secreting (*Sct*), gastric inhibitory polypeptide (*Gip*), cholecystokinin (*Cck*), glucagon (*Gcg*), peptide YYY *(Pyy*) and chromogranin A (*ChgA*). Although the fold-change of EEC markers was lower in the distal SI epithelial cells, their expression was also significantly upregulated after the AB treatment (Fig. 4F). Additionally, comparison of our data set to previously published single-cell RNA sequencing gene sets from sorted EpCAM positive intestinal epithelial cells of adult C57BL/6 wild-type (GEO GSE92332) [88] confirmed that adult enterocytes markers were significantly enriched in distal SI epithelial cells of AB-treated pups compared to control and adult enteroendocrine cell markers were significantly enriched in proximal SI epithelial cells of AB-treated pups compared to control (Fig. 4G and 4H).

Together, these data demonstrate that AB in early life causes gene expression changes specifically in the proximal and distal small intestine, which are associated with intestinal epithelial maturation, intracellular digestion, (innate) defense, and (trans)epithelial sensing functions of the intestinal epithelial cells.

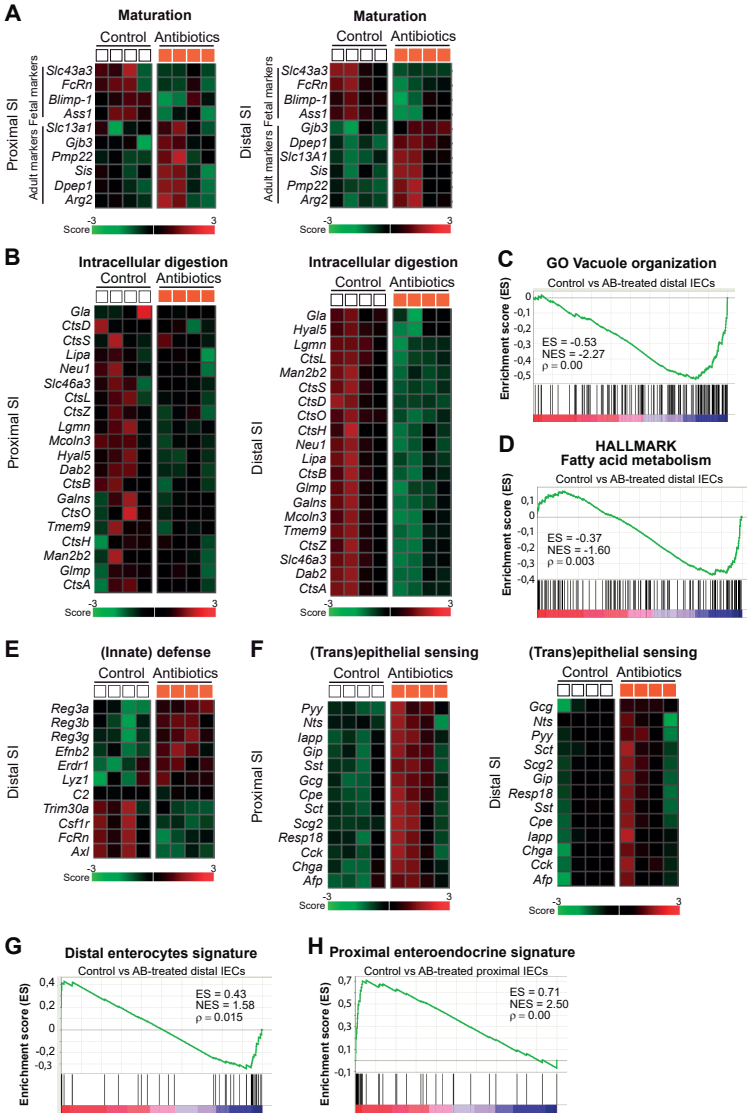


Figure 4. Differential gene expression analysis and gene set enrichment analysis (GSEA) of sorted intestinal epithelial cells after antibiotic treatment.

(**A** and **B**) Curated heatmaps of selected genes from top downregulated and top upregulated genes, based on biological interest and grouped according to function "maturation" (**A**) and "intracellular digestion" (**B**) in proximal and distal epithelial cells. The colored bar represents the expression level from low (green) to high (red). (**C** and **D**) GSEA plots comparing control and antibiotic-treated distal SI epithelial cells against gene sets of Gene Ontology (GO) Vacuole organization (**C**) and HALLMARK Fatty acid metabolism (**D**). Enrichment score (ES), normalized enrichment score (NES), and p values are indicated in the image. (**E** and **F**) Curated heatmaps of selected genes from top downregulated and top upregulated genes, based on biological interest and grouped according to function "(innate) defense" (**E**) in distal epithelial cells and (trans) epithelial sensing (**F**) in proximal and distal epithelial cells. The coloured bar represents the expression level from low (green) to high (red). (**G** and **H**) GSEA plots comparing distal SI control and antibiotic-treated epithelial cells against published gene set of distal SI enterocytes (**G**) and comparing proximal SI control and antibiotic-treated epithelial cells against proximal SI enteroendocrine cell signature (**H**). Enrichment score (ES), normalized enrichment score (NES), and p values are indicated in the image. Statistical analysis by ANOVA eBayes, $p < 0.05$ cut-off. $n = 4$ samples per group.

Antibiotic treatment in early life leads to precocious maturation of the intestinal epithelium and increased expression of enteroendocrine cell markers *in vivo*

We set out to verify the described changes in global transcription by performing an independent *in vivo* experiment and subsequent whole tissue analysis by qRT-PCR. This analysis confirmed the increase in relative expression of adult marker *Sis*, but not of *Arg2*, in the distal SI of AB-treated pups compared with control pups (Fig. 5A). The relative expression of the neonatal markers *FcRn* and *Ass1* upon AB treatment was significantly reduced in distal SI (Fig. 5A). According to genome-wide gene expression analysis, differences in maturation were most obvious in the distal SI (Fig. 4A). Still, whole tissue qRT-PCR analysis revealed that *Sis* and *Arg2,* but not *FcRn* and *Ass1*, were also significantly increased in proximal SI after AB treatment (Fig. 5A). Besides, immunohistochemical analysis showed that the adult marker Sis was only detected at the villus tips in both proximal and distal SI of AB-treated pups but not in control mice (Fig. 5B). At the same time, expression of Ass1 protein, a marker for neonatal epithelium, was reduced at the villus tips of AB mice, especially in distal SI (Fig. 5C).

In agreement with the results of global expression analyses (Fig. 4B), relative expression assessed by qRT-PCR of the intracellular digestion markers *CtsL*, *CtsZ*, *CtsA*, *Dab2,* and *Mcoln3* was decreased (Fig. 6A) in the distal SI of AB-treated pups compared to control pups. Analysis of whole distal SI by qRT-PCR also showed increased relative expression of the innate defense markers *Reg3*β and *Reg3*ɣ after AB treatment (Fig. 6B), but not of *Lyz1*. Yet, immunohistochemical analysis of Lyz1 in distal SI showed a higher number of lysozyme-1 expressing epithelial cells per crypt in AB-treated pups compared to control pups (Fig. 6C). The number of goblet cells as demonstrated by the detection of mucins using alcian blue and periodic acid-Schiff (AB-PAS) staining remained comparable between both groups (Fig. 6D).

Among the top significantly upregulated genes in proximal SI epithelial cells after AB treatment were genes expressed in enteroendocrine cells (EECs), reflecting increased (trans)epithelial sensing (Fig. 4F). This observation was also confirmed in an independent experiment by qRT-PCR analysis of whole proximal SI tissue for genes *Gip*, *Nts*, *Gcg*, *Pyy, Sst*, and *Sct,* except for *Cck* (Fig. 6E). Overall, the genome-wide gene expression, qRT-PCR, and immunohistochemical data show that early life AB treatment of mouse pups leads to a precocious maturation of the intestinal epithelium and induces expression of enteroendocrine markers in the proximal SI tissue.

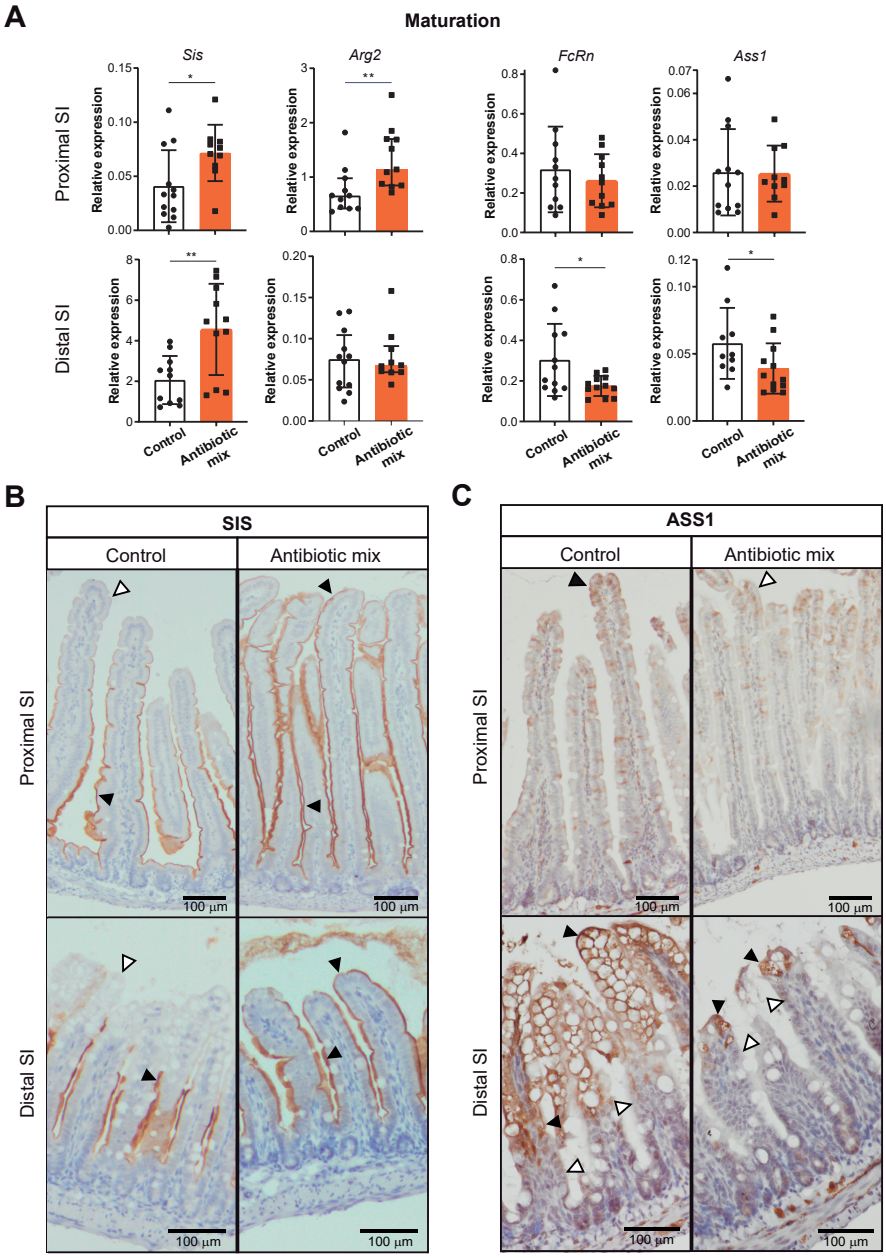


Figure 5. Early life antibiotics induce *in vivo* precocious maturation of the intestinal epithelium.
(**A**) Whole tissue Real-time qPCR analysis of adult maturation markers *Sis* and *Arg2* and of fetal maturation markers *FcRn* and *Ass1* in proximal and distal small intestine. Relative expression to reference genes *Cyp* and *Ppib*. (**B** and **C**) Immunohistochemistry of adult marker Sis (**B**) and fetal marker Ass1 (**C**) in proximal and distal small intestine. Black triangles indicate positive cells and white triangles indicate negative cells. (Scale bars, 100 µm.) Statistical analysis was performed by one-tailed unpaired t-test (*Sis, FcRn,* and *Ass1*) or Mann-Whitney test, as data was not normally distributed when assessed by D'Agostino and Pearson normality test (*Arg2*). Error bars indicate mean ± SD (*Sis, FcRn,* and *Ass1*) or median with interquartile range (*Arg2*). Levels of significance are indicated (*p<0.05, **p<0.01). *n* = 10-12 pups per group.

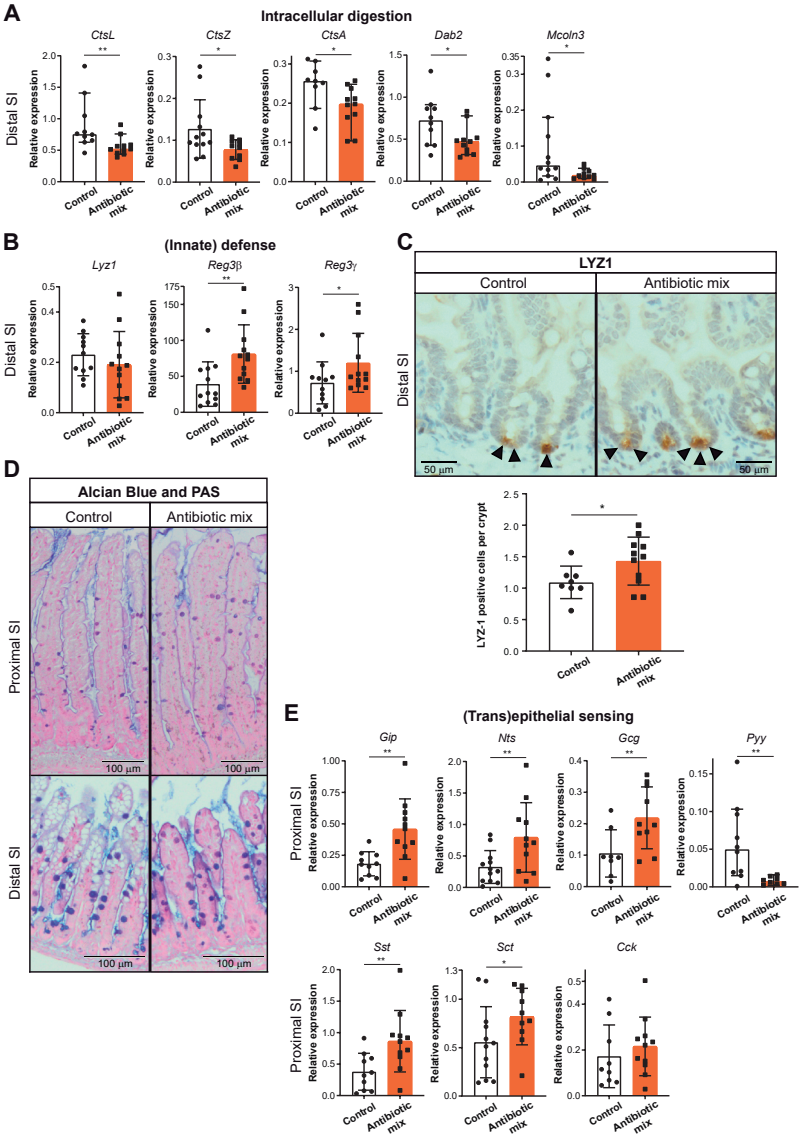


Figure 6. Treatment with early life antibiotics *in vivo* decreases expression of intracellular digestion markers and increases expression of innate defense and enteroendocrine cell markers.

(**A** and **B**) Whole tissue Real-time qPCR analysis of intracellular digestion markers *CtsL, CtsZ, CtsA, Dab2,* and *Mcoln3* (**A**) and of innate defense markers *Lyz1*, *Reg3β,* and *Reg3γ* (**B**) in distal small intestine. (**C**) Immunohistochemistry of LYZ1 and quantification of amount of Lysozyme-1 positive cells per crypt in distal small intestine. (Scale bars, 100 µm.) (**D**) Alcian Blue and PAS staining of proximal and distal small intestine. (Scale bars, 100 µm.) (**E**) Whole tissue Real-time qPCR analysis of (trans)epithelial sensing markers *Gip, Nts, Gcg, Pyy, Sst*, *Sct*, and *Cck* in proximal small intestine. Relative expression to reference genes *Cyp* and *Ppib*. Statistical analysis was performed by one-tailed unpaired t-test (*CtsZ, Dab2, Lyz, Reg3γ, Gip, Nts, Gcg, Pyy, Sst*, *Sct*, and *Cck*) or Mann-Whitney test, as data was not normally distributed when assessed by D'Agostino and Pearson normality test (*CtsL, CtsA, Mcoln3*, and *Reg3β*). Error bars indicate mean ± SD (*CtsZ, Dab2, Lyz, Reg3γ, Gip, Nts, Gcg, Pyy, Sst, Sct*, and *Cck*) or median with interquartile range (*CtsL, CtsA, Mcoln3*, and *Reg3β*). Levels of significance are indicated (*p<0.05, **p<0.01). *n* = 8-12 pups per group.

Mouse intestinal fetal organoids reveal the direct effects of early life antibiotics on intestinal epithelial maturation and differentiation of enteroendocrine cells

Intestinal epithelial maturation can be recapitulated *in vitro* (during a course of one month), using mouse fetal intestinal organoids [80]. To study whether the changes in intestinal epithelial maturation we observed *in vivo* are a direct effect of the AB on intestinal epithelial cells, we used fetal intestinal organoids from embryonic stage 19 (E19). We separately cultured the proximal and distal SI fetal organoids in the presence or absence of the same AB mix (amoxicillin, vancomycin, and metronidazole) as used *in vivo* (Fig. 7A). Immature fetal organoid cultures present more spherical organoids [89, 90], which are replaced over time by budding organoids as the cells mature in culture [80]. The appearance of proximal and distal SI organoids was evidentially different, with less spherical organoids and more budding organoids observed in the culture treated with AB compared to control (Fig. 7B). Indeed, AB-treated organoids presented higher number of budding structures compared to control organoids (Fig. 7C).

Comparable to our *in vivo* observations, gene expression analysis by qRT-PCR showed a significant upregulation of adult markers *Sis* and *Arg2* by AB treatment in proximal SI fetal organoids, and of *Sis* also in distal SI fetal organoids, after 20 days of culture (Fig. 8A). Accordingly, Sis protein levels were significantly increased by AB treatment in both proximal and distal SI organoids, as detected by immunohistochemistry (Fig. 8B). The relative expression of neonatal markers *FcRn* and *Ass1* was not affected by the AB treatment *in vitro* (Fig. 8C). There was no effect of the AB treatment *in vitro* on the gene expression level of *CtsL*, CtsZ, CtsA, *Dab2,* and *Mcoln3* in distal SI organoids, in contrast to what was demonstrated *in vivo* (Fig. 8D).

The Paneth cell marker *Lyz1* was upregulated in whole-genome expression analysis of distal IECs of the AB treated group (Fig. 4E). *In vitro* observations confirmed the significant increase in *Lyz1* expression after AB treatment in distal SI fetal organoids (Fig. 9A), which was further supported by staining for the Lyz1 protein (Fig. 9B). In contrast to Lyz1, the other markers of innate defense, *Reg3*β and *Reg3*γ, did not show the consistent increased gene expression levels in distal SI AB-treated organoids as upon AB treatment *in vivo* (Fig. 9C). Similar to our *in vivo* findings, proximal SI organoids treated with AB exhibited a significant increase in the expression of EEC markers *Gip*, *Nts*, *Gcg*, and *Pyy*, but not of the expression of *Sst*, *Sct,* and *Cck* (Fig. 9D). To further examine this, expression of GIP was analyzed by immunostaining of proximal SI organoids which revealed a greater number of GIP-positive cells in proximal SI AB-treated organoids compared to control organoids (Fig. 9E). In summary, these results indicate that AB can partially, but directly, affect the maturation of intestinal epithelial cells and differentiation of enteroendocrine cells.

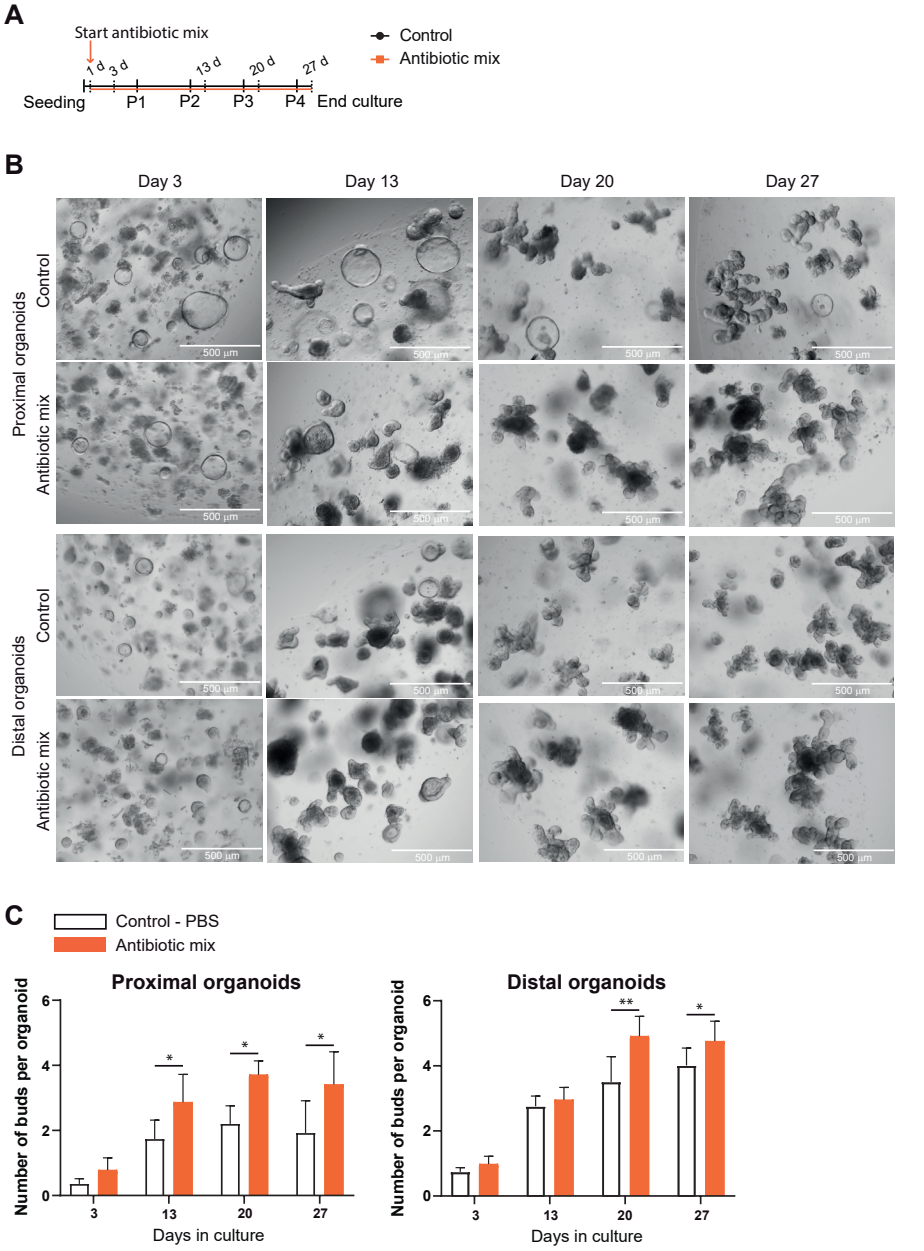


Figure 7. Appearance and budding quantification of proximal and distal fetal intestinal organoids treated with early life antibiotics over time.

(**A**) Experimental design of *in vitro* treatment of mouse fetal intestinal organoids with the antibiotic mix. Organoids were analyzed at 3, 13, 20, and 27 days of culture. (**B**) Microscopic images of control and antibiotic mix-treated organoids at day 3, 13, 20, and 27 of culture. (Scale bars, 500 µm.) (**C**) Quantification of the number of buds per organoid of proximal and distal cultures in control and antibiotic mix conditions. Statistical analysis was performed by one-tailed paired t-test. Error bars indicate mean ± SD. Levels of significance are indicated (*p<0.05, **p<0.01). *n* = 18 to 57 organoids per condition and day of 4 independent cultures.

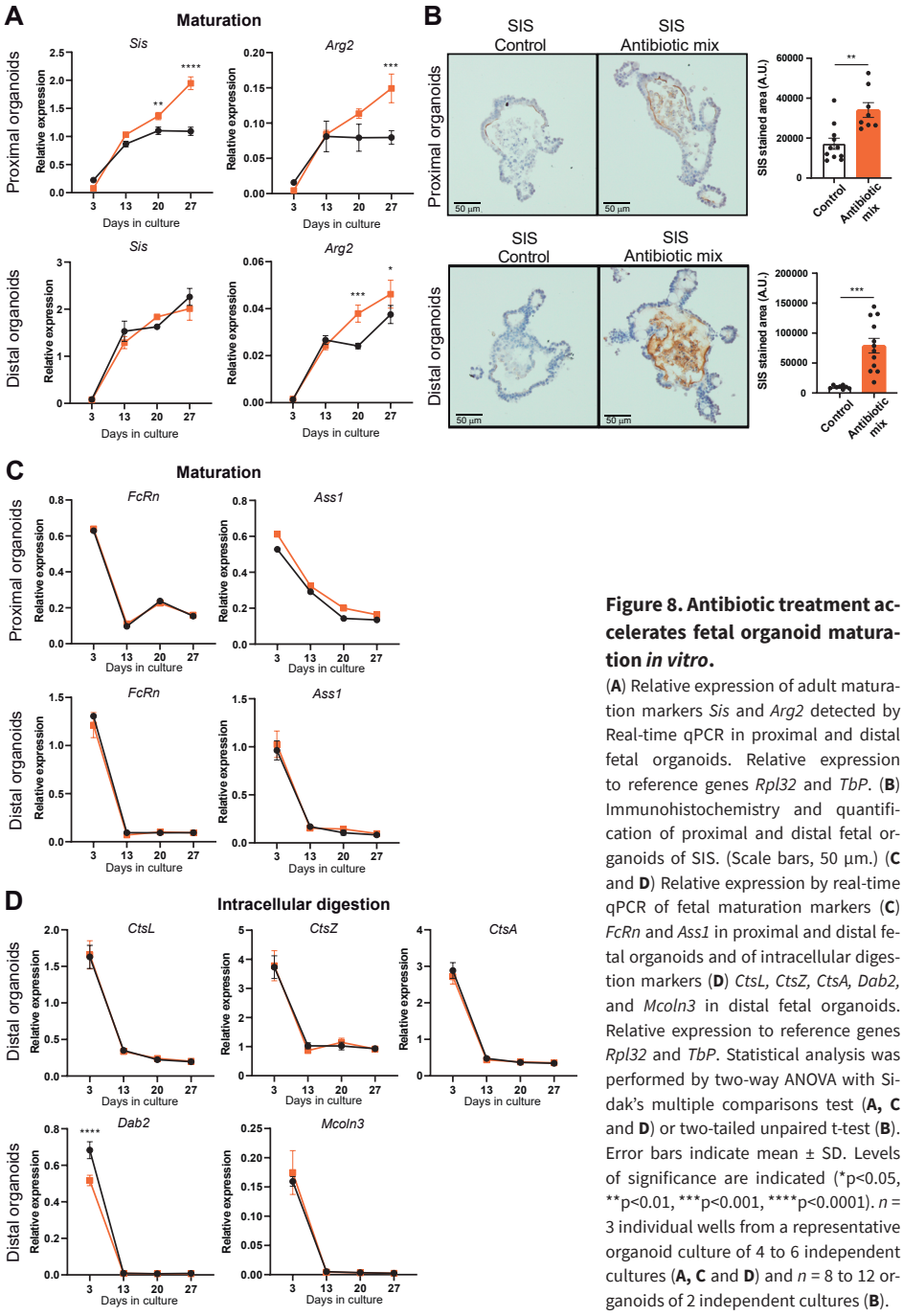


Figure 8. Antibiotic treatment accelerates fetal organoid maturation *in vitro*.

(**A**) Relative expression of adult maturation markers *Sis* and *Arg2* detected by Real-time qPCR in proximal and distal fetal organoids. Relative expression to reference genes *Rpl32* and *TbP*. (**B**) Immunohistochemistry and quantification of proximal and distal fetal organoids of SIS. (Scale bars, 50 µm.) (**C** and **D**) Relative expression by real-time qPCR of fetal maturation markers (**C**) *FcRn* and *Ass1* in proximal and distal fetal organoids and of intracellular digestion markers (**D**) *CtsL, CtsZ, CtsA, Dab2,* and *Mcoln3* in distal fetal organoids. Relative expression to reference genes *Rpl32* and *TbP*. Statistical analysis was performed by two-way ANOVA with Sidak's multiple comparisons test (**A, C** and **D**) or two-tailed unpaired t-test (**B**). Error bars indicate mean ± SD. Levels of significance are indicated (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). *n* = 3 individual wells from a representative organoid culture of 4 to 6 independent cultures (**A, C** and **D**) and *n* = 8 to 12 organoids of 2 independent cultures (**B**).

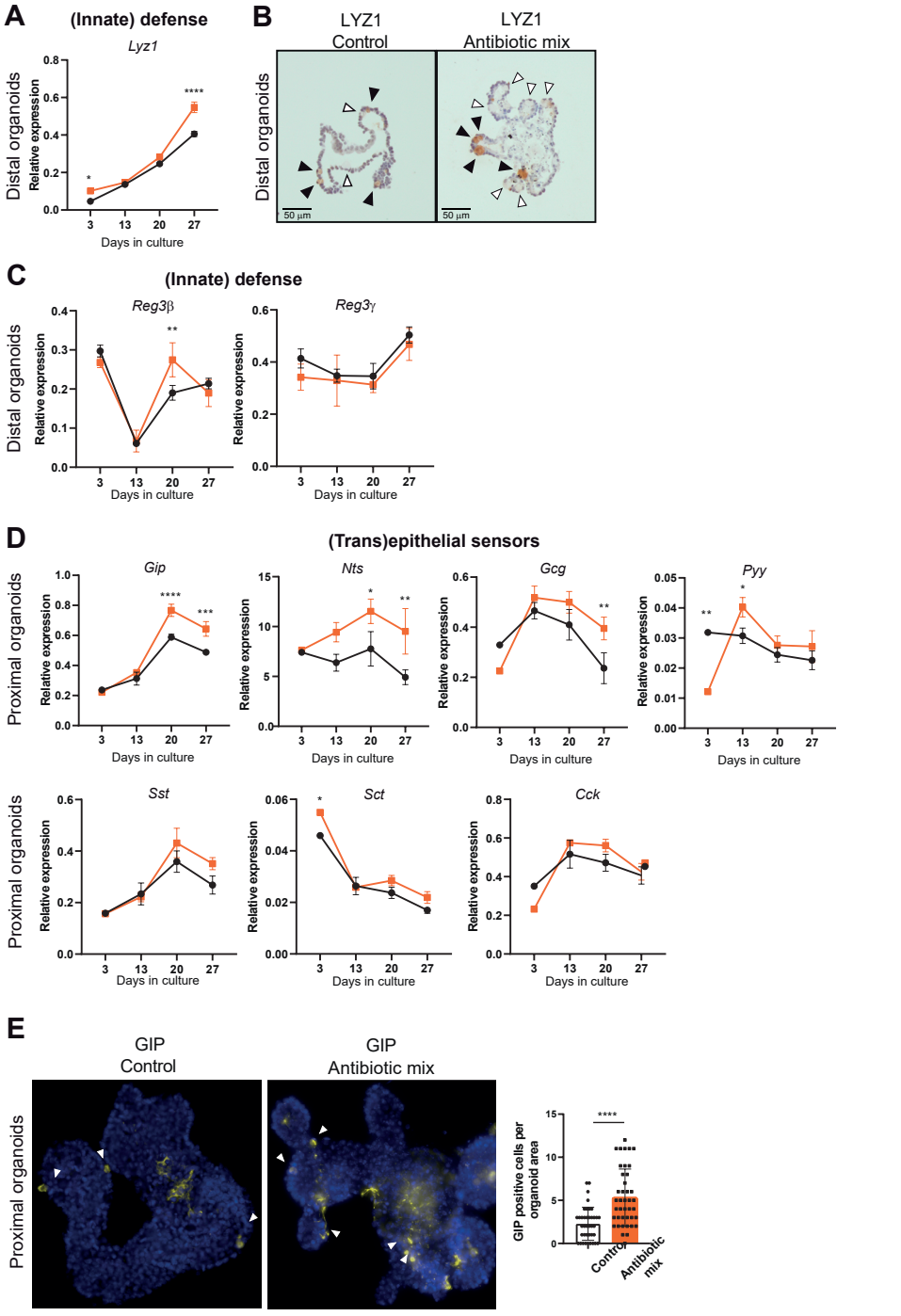


Figure 9. Antibiotic treatment of *in vitro* fetal organoids induces differentiation of Paneth cells and enteroendocrine cells.

(continued) Figure 9. Antibiotic treatment of *in vitro* fetal organoids induces differentiation of Paneth cells and enteroendocrine cells.
(**A** and **B**) Real-time qPCR analysis (**A**) and immunohistochemistry (**B**) of innate defense marker *Lyz1*/LYZ1 in distal fetal organoids. Relative expression to reference genes *Rpl32* and *TbP*. (Scale bars, 50 μm.) (**C** and **D**) Real-time qPCR analysis of innate defense markers (**C**) *Reg3β* and *Reg3γ* in distal fetal organoids and (trans)epithelial sensing markers (**D**) *Gip, Nts, Gcg, Pyy*, *Sst, Sct*, and *Cck* in proximal fetal organoids. Relative expression to reference genes *Rpl32* and *TbP*. (**E**) Immunofluorescence of whole proximal fetal organoids for GIP and quantification of the amount of GIP positive cells per organoid area. Statistical analysis was performed by two-way ANOVA with Sidak's multiple comparisons test (**A**, **C** and **D**) or two-tailed unpaired t-test (**E**). Error bars indicate mean ± SD. Levels of significance are indicated (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). *n* = 3 individual wells from a representative organoid culture of 4 to 6 independent cultures (**A**, **C**, and **D**) and *n* = 40 organoids of 2 independent cultures (**G**).

Antibiotics directly reduce metabolic capacity of fetal intestinal organoids

Several metabolic genes were changed in whole-genome expression analysis of IECS upon *in vivo* AB treatment (Table S1 and S2). Additionally, different cells in the intestine have different metabolic profiles [91]. To test whether AB disturb the oxidative phosphorylation (or respiration) capacity of neonatal intestinal epithelial cells, we measured the real-time oxygen consumption of fetal organoids treated with the same mix of early life AB (amoxicillin, vancomycin, and metronidazole) for 96 hours (Fig. 10A). Oligomycin, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP), and Rotenone together with Antimycin (Rot+Ant) were added sequentially to the organoids in order to challenge the mitochondria and determine the amount of ATP production and maximal capacity of mitochondrial respiration (Fig. 10B). The oxygen consumption rate of proximal SI organoids was not affected by AB (Fig. 10C). Distal SI fetal organoids showed significantly lower basal respiration and impaired capacity for maximal respiration when treated with AB, while ATP production followed the same trend (Fig. 10D).

Next, we assessed real-time changes in extracellular acidification, a measurement of glycolysis in cells, to understand whether decreased respiration as a result of early life AB is compensated for by an increase in glycolysis. Sequential addition of glucose, oligomycin, and 2-deoxy-glucose (2-DG) to the organoids allows the quantification of glycolysis rate and maximal glycolytic capacity (Fig. 11A). Interestingly, we found that AB treatment significantly limited glycolysis in distal SI organoids and limited glycolytic capacity in both proximal and distal SI organoids (Fig 11B and 11C). Together, our results demonstrate that AB treatment of fetal gut organoids directly affects the cellular metabolism of intestinal epithelial cells.

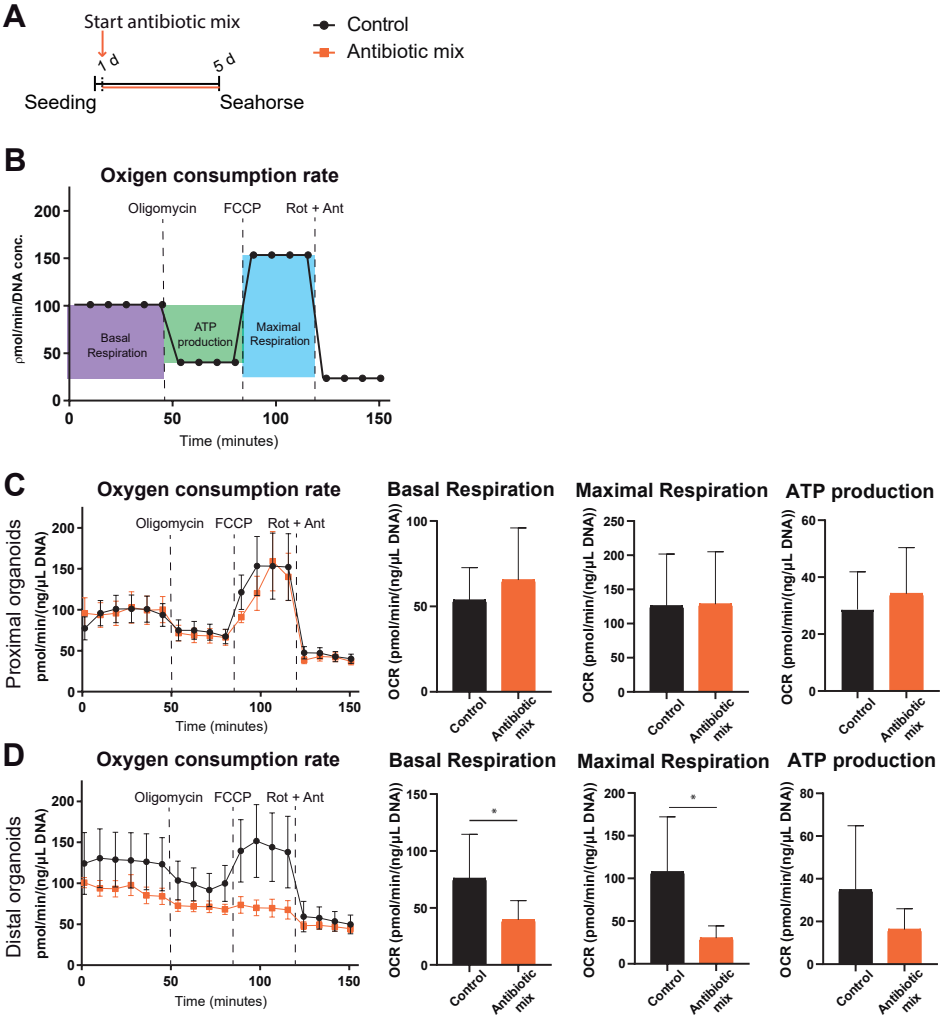


Figure 10. Respiration capacity of distal fetal organoids decreases upon *in vitro* early life antibiotics. (**A**) Experimental design of seahorse experiments to measure mitochondrial respiration and glycolysis in mouse fetal intestinal organoids after 5 days of antibiotic mix treatment. (**B**) Graphic representation of key parameters measured by oxygen consumption rate (OCR). (**C** and **D**) Real-time respiration levels in the supernatant of proximal (**C**) and distal (**D**) fetal organoids measured as oxygen consumption rate (OCR), and basal respiration, ATP production, and maximal respiration rates calculated using OCR levels determined by seahorse assay. Statistical analysis was performed by one-tailed unpaired t-test. Error bars indicate mean ± SEM (line graphs) or mean ± SD (column graphs). Levels of significance are indicated (*p<0.05). *n* = 4-5 individual wells, representative of 2-4 independent experiments.

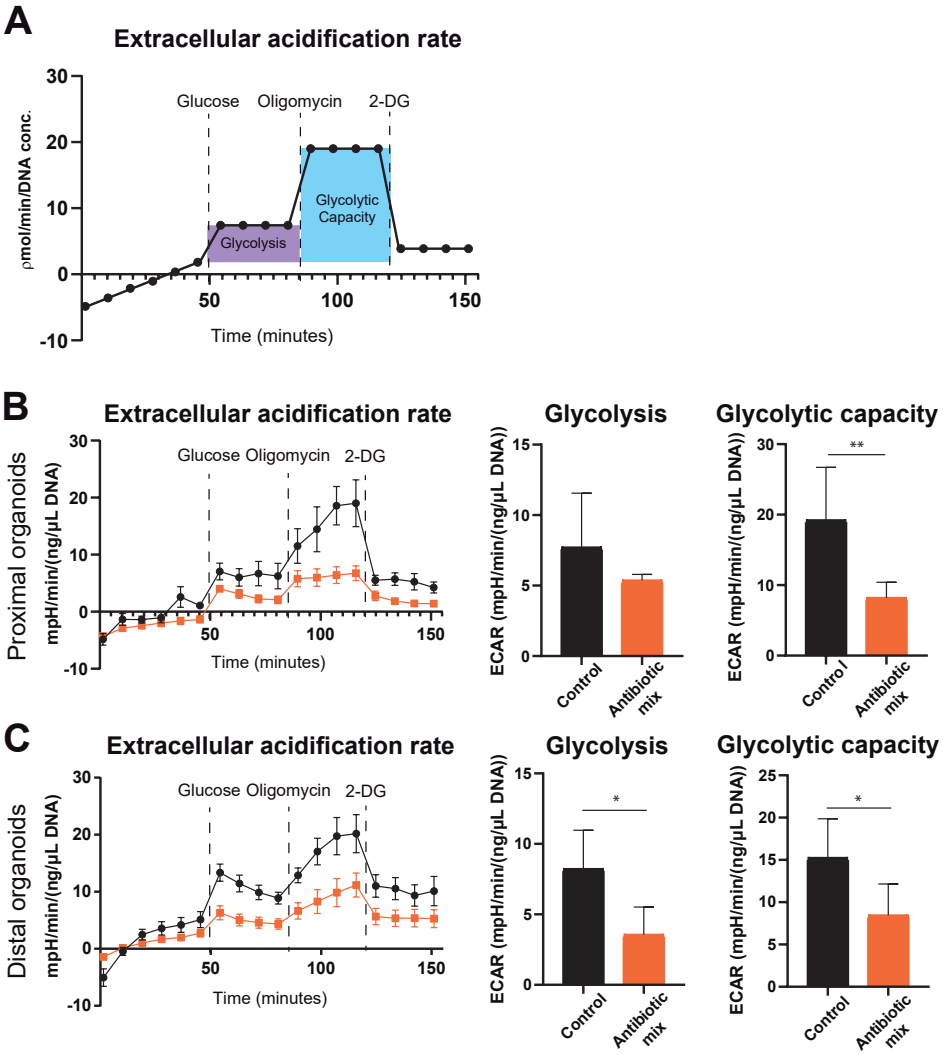


Figure 11. Glycolytic capacity of fetal organoids decreases upon *in vitro* early life antibiotics.
(**A**) Graphic representation of key parameters measured by extracellular acidification rate (ECAR). (**B** and **C**) Real-time glycolysis levels in the supernatant of proximal (**B**) and distal (**C**) fetal organoids measured as extracellular acidification rate (ECAR), and glycolysis and glycolytic capacity calculated using ECAR levels determined by seahorse assay. Statistical analysis was performed by one-tailed unpaired t-test. Error bars indicate mean ± SEM (line graphs) or mean ± SD (column graphs). Levels of significance are indicated (*p<0.05, **p<0.01). *n* = 4-5 individual wells, representative of 2-4 independent experiments.

DISCUSSION

In this study, we showed that AB usage during early life induces various changes in neonatal intestinal epithelial cells. We found that AB exposure accelerates the maturation of the suckling intestinal epithelium, demonstrated by decreased gut permeability, the disappearance of vacuolated enterocytes, downregulation of fetal/neonatal markers, and upregulation of adult and (innate) defense markers (Fig. 2 and 4). We observed decreased gut permeability upon AB treatment in contrast to several previous studies that reported increased gut permeability after AB treatment [44, 55, 92, 93]. These studies were performed in adult mice, indicating that the developmental stage of the intestinal epithelium is an important factor determining the effect of the AB. The reduced permeability we observed in the neonatal period could be due to the loss of vacuolated enterocytes, since the ACS contained in these cells can transport the FITC-dextran from the intestinal lumen to the basolateral side [94, 95]. ACS are responsible for the intact transfer of milk immunoglobulins through the proximal intestinal epithelium [68, 69]. We could not find a difference in IgG and IgA serum levels upon AB treatment. The vacuolated cells are replaced by adult epithelium from the proximal SI enterocytes around P15 [65, 69] while they persist in the distal SI until weaning (P21) [96], following a proximal to distal maturation wave [63]. In our study, serum was analyzed at P20, which might represent a suboptimal time point to detect the differences in Ig uptake, as by then the vacuolated cells, responsible for immunoglobulin transfer, have already disappeared from the proximal SI. Nevertheless, our data show that the uptake of milk macromolecules by the intestinal epithelium of AB-treated mice is limited. Further studies with long-term follow-up are required to confirm whether precocious loss of vacuolated enterocytes has consequences in adult life or under disease conditions.

Global gene expression analyses showed a clear differential gene expression in the small intestine epithelium between control and AB-treated pups (Fig. 3 and 4). The fact that greater differences were found in the distal SI compared to proximal SI might be due to the higher density of microbiota in the distal SI. AB can strongly deplete the distal SI microbes, but some strains will survive the treatment, re-populate the intestinal lumen, and differently influence the intestinal epithelial cells. Additionally, due to the proximal-to-distal wave of epithelial maturation that occurs during the suckling-to-weaning transition, proximal maturation is mostly completed by P20. Importantly, several differences in global gene expression were confirmed by qRT-PCR, and immunostaining, in an independent experiment (Fig. 5 and 6). Collectively, these results indicate precocious maturation of the intestinal epithelium after treatment with early life AB. Increased sensitivity to induced colitis in mice has been described after AB treatment [37, 45, 48, 52, 92]. Recently, Al Nabhani *et al.* demonstrated that metronidazole and vancomycin

depleted bacterial strains essential for the induction of the weaning reaction [48]. Here, we provide evidence that AB treatment not only disturbs the development of the microbiota, and, consequently, the development of the immune system, but also affects the maturation process of the intestinal epithelial cells, which can contribute to the negative long-term effects. Moreover, our *in vitro* studies on fetal organoids showed that acceleration of epithelial maturation is partially a result of the direct action of AB on the epithelial cells, indicated by the increased expression of adult maturation markers and Paneth cell marker lysozyme-1 (Fig. 7 to 9). These findings confirm our previous study in which we demonstrated that the intrinsic maturation process of the intestinal epithelium can be modulated by external factors, such as early life AB as demonstrated here.

In this study, we demonstrated the upregulation of enteroendocrine cell markers after AB treatment in early life is a direct effect of AB (Fig. 6 and 9). Enteroendocrine cells are pivotal for (trans)epithelial sensing of specific nutrients, microbiota structures, and metabolites in the gut and for the translation of these signals in the modulation of specific processes, such as the release of pancreatic, gastric and hepatic enzymes, glucose homeostasis, food intake, and intestinal motility. Previous studies also demonstrate increased serum EEC-produced hormones GLP-1 (glucagon-like peptide-1), GCG, and GIP levels in adult mice treated with AB [35, 46] and an upregulated expression of *Gcg, Pyy,* and *Cck* in the cecum [46]. Zarrinpar *et* al. attributed these changes to an indirect effect of AB, through the microbiota, on the epithelial cells [46]. However, in our fetal organoid model, the increase in expression of the enteroendocrine cell markers suggests that, in the neonatal epithelium, the effect of AB on the expression of specific EEC markers is at least partly a direct effect. The increased (trans)epithelial sensing and altered lipid metabolism detected in our global gene expression analysis (Table S1 and S2) could be involved in the increased risk of developing obesity and diabetes later in life after (prolonged) use of AB in early life [31-35]. Treatment of our fetal organoid model with AB also showed that part of their effect was indirect. We observed no change in intracellular digestion and innate defense (except for lysozyme), nor for a couple of maturation and (trans)epithelial sensing markers, as identified by our *in vivo* approach. This indicates that many effects of early life AB on intestinal epithelial cells are due to alterations in the microbiome.

Finally, this study provides further evidence that AB can directly disturb intestinal epithelial cell metabolism (Fig. 10 and 11), supporting previous studies. Morgun *et al.* demonstrated that several intestinal genes affected by AB treatment in germ-free mice were similarly changed upon AB treatment of conventional mice [57]. These genes, identified as a result of the direct effect of AB treatment, were mainly expressed in the epithelium and belonged to mitochondrial gene categories (electron transport chain,

oxidation-reduction, ATP biosynthesis, cellular and mitochondrial ribosomes) [57]. Moreover, the number of mitochondria in intestinal epithelial cells treated with AB was reduced [57], which supports the concept that mitochondria, being structurally and functionally similar to bacteria, are a target of AB. The decreased (maximal) respiration measured in distal SI fetal organoids is in agreement with earlier reports showing diminished mitochondrial membrane potential and functions in cell lines [56, 97] upon AB treatment. Ampicillin, belonging to the same class and with the same target as amoxicillin, reduces mitochondrial basal respiration and maximal respiratory capacity, as well as overall metabolic activity in the intestinal epithelial cell line Caco-2 [56]. Vancomycin has been shown to depolarize the mitochondrial membrane, inhibit the mitochondrial complex I activity, and induce the production of mitochondrial reactive oxygen species in renal and kidney epithelial cells [98-100]. Additionally, we found a reduction in glycolytic rate in distal SI organoids after AB treatment and reduced glycolytic capacity in AB-treated proximal and distal SI organoids. The decreased oxygen concentration along the proximal-to-distal small intestine [101] and the changed expression of glucose transporters in the distal SI of AB-treated pups (Table S2) might partly be responsible for the observed stronger effect of AB on distal epithelial cells. The combination of reduced expression of *Slc2a2*/*Glut2*, that transports glucose at the basolateral membrane, downregulation of *Slc37a4*, which is involved in the release of glucose to the blood upon glycogenolysis and gluconeogenesis, and upregulation of *Slc2a1/Glut1*, responsible for basolateral uptake of glucose into the cell, seems to indicate that distal cells have limited access to glucose, leading to lower metabolic capacity. Therefore, the most probable mechanism for the observed decrease in intestinal metabolism upon AB treatment is the vancomycin-driven inhibition of mitochondrial complex I, together with the reduced capacity of glucose uptake. Furthermore, it was previously reported that adult intestinal organoids have lower glycolytic capacity compared to Wnt-induced fetal organoids [91]. The lower glycolytic rate induced by AB treatment of the fetal organoids might drive the precocious maturation we observed. More detailed studies are necessary to confirm this hypothesis and identify the possible mechanism.

Necrotizing enterocolitis (NEC) is one of the diseases associated with early life AB [23-25]. This severe disease, with long-term sequelae and mortality rates between 10% to 50%, is thought to frequently affect preterm infants because of their immature intestine [102]. The accelerated and inappropriate maturation of the intestinal epithelium upon AB, as well as the increased and inadequate (innate) intestinal defense, might explain the association between AB and NEC in preterm infants. Further studies may elucidate which of the different types and combinations of AB is responsible for the changes described in our study. Restricting the use to a single type or class, using different AB

combinations, or adjust the duration of treatment, may reduce the risk of diseases, such as NEC, and prevent later disease development.

In conclusion, our study contributes to a better understanding of how AB administration early in life can indirectly and directly affect intestinal epithelial cells. Future investigations are needed to elucidate underlying mechanisms, in order to maximize the relevance and translational value of our findings.

METHODS

In vivo studies

This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Animal Ethics Committee of the University of Amsterdam, and all animal procedures related to the purpose of the research were approved under the Ethical license of the national competent authority, securing full compliance the European Directive 2010/63/EU for the use of animals for scientific purposes.

Six pregnant 8 weeks old C57Bl/6J females were obtained from Charles River and were allowed to adapt to the new environment for 1 week. Pregnant females were individually housed and received AIN-93G diet (Triple A Trading/Altromin). Mice were kept in Innovive disposable mice cages, enriched with corncob bedding and carton house, with tissue as nesting material, under a strict 7 am to 7 pm dark-light cycle, controlled temperature and humidity, with food and water given *ad libitum*. Pups were monitored daily, weighted every other day from P10 onwards, and kept with the mothers throughout the experiment. At P10, two experimental groups were randomly defined: treatment group (3 litters, 4 pups per litter) received daily oral gavage of 30µl of AB (25mg/kg/day amoxicillin (Amsterdam UMC, AMC pharmacy), 50mg/kg/day metronidazole (Amsterdam UMC, AMC pharmacy) and 50mg/kg/day vancomycin (Sigma/Aldrich)); control group (3 litters, 4 pups per litter) received daily oral gavage of 30µl of PBS. AB or PBS were consistently given during the light period, always at the same time period of the day, nonblinded. For oral gavage, pups were separated from the mother all at once and placed back all at once as well, to correct for differences in maternal care. On P20, mothers were euthanized by CO_2 and isoflurane exposure and pups were fasted. After 3 hours, 50µl of 60mg/100gr weight FITC-dextran 4kDa (Sigma/Aldrich) diluted in PBS were given via oral gavage to all the pups, to assess intestinal permeability. After 4 hours, pups were euthanized by CO_2 and isoflurane exposure. Immediately after, blood was collected by heart puncture

in MiniCollect® Z Serum Sep Clot tubes (Greiner). After 30 minutes of incubation on ice, in the dark, blood was centrifuged and serum was collected and kept at -80°C.

FITC-dextran *in vivo* permeability assay

Standard samples were obtained by 2-fold serial dilution of 1mg/ml FITC-dextran in blood serum. The fluorescence signals of the serum samples were recorded with an excitation wavelength of 485 nm and an emission wavelength of 520 nm and compared with the standard curve values. The amount of FITC-dextran in serum samples was calculated in ng/ml.

In vitro studies

Per experiment, two pregnant 8 weeks old C57Bl/6J mice were obtained from Charles River, housed together, and were euthanized by CO_2 and isoflurane exposure on day 19 of the pregnancy. To generate fetal organoid culture, fetuses from 2 mice were combined resulting in a final number of around 12-15 fetuses per experiment. Fetal small intestine tissue was harvested, separated into proximal and distal parts, dissociated, and cultured as previously described in a 48-well plate [80, 81]. Fetal organoids were maintained in ENR medium, without penicillin and streptomycin, throughout the experiments. On day 1 of culture, 15 µg/mL amoxicillin (Amsterdam UMC, AMC pharmacy), 25 µg/mL metronidazole (Amsterdam UMC, AMC pharmacy), and 50 µg/mL vancomycin (Sigma/Aldrich) were added to half of the wells. The medium was refreshed 3 times per week. Samples for RNA analyses or immunostaining were always taken 3 days after passaging of the culture. Representative images of the cultures were taken by an inverted light microscope (Leica).

Immunostaining

Tissue was flushed with PBS, fixed overnight in 4% formaldehyde, embedded in paraffin, and sectioned. Sections were deparaffinized with xylene and gradually rehydrated in ethanol. After blocking the endogenous peroxidase (0,01% H2O2 in methanol; only performed for immunohistochemistry), slides were boiled in 0,01M sodium citrate buffer (pH 6) for 10 minutes at 120˚C in an autoclave for antigen retrieval. Slides were blocked for 30 minutes at room temperature in PBS with 1% bovine serum albumin and 0,1% Triton-X-100. Then, slides were incubated overnight with primary antibody diluted in the blocking buffer. Slides were washed with PBS and secondary antibody diluted in blocking buffer was added for 30 minutes at room temperature. For immunofluorescence, slides were mounted using Prolong Gold antifade reagent with DAPI (Invitrogen). For immunostaining, antibody binding was visualized by adding chromagene substrate diaminobenzidine (Sigma-Aldrich), sections were counterstained using hematoxylin (Sigma) and slides were dehydrated and mounted with Entellan.

Immunohistochemistry in fetal organoids was performed as previously described [81, 103]. For whole-mount immunofluorescence staining, organoids were collected from the matrigel by Cell Recovery Solution (Corning B.V.) and fixed for 1 hour in 4% formaldehyde. After washing (PBS + glycine), permeabilization (PBS + 0.5% Triton X-100), and blocking (IF-wash + 10% goat serum), organoids were incubated with primary antibody diluted in blocking buffer for 1-2 hours at room temperature. Staining was visualized with Alexa-conjugated secondary antibody (1 hour at room temperature) after which cells were mounted on a slide with ProLongTM Gold antifade reagent with DAPI (Invitrogen). Sections were examined using brightfield microscope Olympus BX51 or fluorescence microscope Leica DM-6000B.

Ki67 – rabbit polyclonal anti-Ki67 (1:4000, Abcam, ab15580)
Phospho-Histone H3 – rabbit polyclonal anti-phospho-Histone H3 (Ser10) (1:200, ThermoFisher Scientific, PAS-17869)
SIS – rabbit monoclonal anti-sucrase isomaltase (C-8) (1:200, Santa Cruz, sc-393470)
ASS1 – rabbit polyclonal anti-argininosuccinate synthethase I (1:15000, [104]
LYZ1 – rabbit polyclonal anti-lysozyme (1:2000, DAKO, A0099)
GIP – rabbit polyclonal anti-gastric inhibitory peptide (1:500, Abcam, ab22624)
AF488 - goat polyclonal anti-rabbit (1:500, Invitrogen, A11008)

Immunoglobulins detection

Performed in serum samples using IgG and IgA total ELISA Ready-SET-Go!® kit (Affymetrix eBioscience) and according to the manufacturer's protocol.

Epithelial cells FACS-sorting

The small intestine of P20 pups was cut open and proximal and distal parts were separated, cut into pieces, and washed with ice-cold PBS. Intestines of 4 different pups, belonging to the same litter, were combined into one sample used for transcriptome profile analysis. Crypts were dissociated after incubation with 2 mM EDTA (Merck/VWR) for 30 minutes at 4°C and filtered through a 70 µm cell strainer (BD/VWR). Single cells were obtained by incubating crypts with TrypLE Express (Invitrogen). Cells were kept in PBS 2% FCS Rho-kinase inhibitor and RNase inhibitor (Fermentas/Thermo Fisher Scientific) solution and stained with EpCAM-FITC antibody (1:50, BioLegend, 324204) for 30 minutes on ice.

RNA isolation and qRT-PCR

For transcriptome profiling, RNA was extracted from EpCAM-positive cells using the phenol-chloroform method. RNA quality was measured on an Agilent 2100 Bioanalyzer.

For qRT-PCR, RNA from whole-tissue tissue and fetal organoids was isolated using the Bioline ISOLATE II RNA Mini kit (BIO-52073, Bioline) according to manufacturers' instructions. 1 µg (tissue) or 0,3 µg (organoids) of RNA was transcribed using Revertaid reverse transcriptase according to the protocol (Fermentas, Vilnius, Lithuania). Quantitative RT-PCR was performed on a BioRad iCycler using sensifast SYBR No-ROX Kit (GC-biotech Bio-98020) according to the manufacturer's protocol. The 2 most stable reference genes were determined using GeNorm and their geometric mean was used to calculate the relative expression of genes of interest: for whole-tissue qRT-PCR cyclophilin (*Cyp*) and peptidylprolyl isomerase B (*Ppib*) and for fetal organoids qRT-PCR ribosomal protein L32 (*Rpl32*) and TATA-Box Binding Protein (*TbP*). Relative gene expression was calculated using N0 values obtained by LinRegPCR analysis. Primers were previously validated using melting curve analyses and gel electrophoresis of PCR products.

Cyp: FW - ATGGTCAACCCCACCGTGT; RV - TTCTGCTGTCTTTGGAACTTTGTC
Ppib: FW – GCCAACGATAAGAAGAAGGGA; RV - TCCAAAGAGTCCAAAGACGAC
Tbp: FW – GGGTATCTGCTGGCGGTTT; RV - TGGAAGGCTGTTGTTCTGGT
Rpl32: FW – TGGAGGTGCTGCTGATGTG; RV - GCGTTGGGATTGGTGACTCT
Sis: FW – TGCCTGCTGTGGAAGAAGTAA; RV - CAGCCACGCTCTTCACATTT
Arg2: FW – TAGGGTAATCCCCTCCCTGC; RV - AGCAAGCCAGCTTCTCGAAT
FcRn: FW – CTTCAGGCGCATAGACGG; RV - CTAAACTCTTGTCCGGAGCG
Ass1: FW – CATTGGAATGAAGTCCCGAG; RV - GATTTTGCGTACTTCCCGAT
CtsL: FW – CGACTGTGGGGCCTATTTCT; RV - ATAGCCCACCAACAGAACCC
CtsZ: FW - CTACCAGGCCAAGGACCAAG; RV - GCCATTATCCCGCAGCTGAT
CtsA: FW - GCTAGTGGACTACGGGGAGA; RV - GTGTCCGGCACCCTTGATG
Dab2: FW - TCATCAAACCCCTCTGTGGT; RV - AGCGAGGACAGAGGTCAACA
Mcoln3: FW - TTTTGCGGATGGATTGTGCT; RV - TATCAGCGAGAACAGGCACTC
Lyz: FW – GGATGGCTACCGTGGTGTCAAGC; RV - TCCCATAGTCGGTGCTTCGGTC
Reg3β: FW – TGGGAATGGAGTAACAAT; RV - GGCAACTTCACCTCACAT
Reg3γ: FW - CCATCTTCACGTAGCAGC; RV - CAAGATGTCCTGAGGGC
Gip: FW – AACTGTTGGCTAGGGGACAC; RV - TGATGAAAGTCCCCTCTGCG
Nts: FW – TGCTGACCATCTTCCAGCTC; RV - GAATGTAGGGCCTTCTGGGT
Gcg: FW – CTTCCCAGAAGAAGTCGCCA; RV - GTGACTGGCACGAGATGTTG
Pyy: FW – ACGGTCGCAATGCTGCTAAT; RV - GCTGCGGGGACATCTCTTTTT
Sst: FW – GACCTGCGACTAGACTGACC; RV - CCAGTTCCTGTTTCCCGGTG
Sct: FW – GACCCCAAGACACTCAGACG; RV - TTTTCTGTGTCCTGCTCGCT
Cck: FW – GAAGAGCGGCGTATGTCTGT; RV - CCAGAAGGAGCTTTGCGGA

Transcriptome profiling

For transcriptome profiling, 400ng RNA was amplified and labelled using 3' IVT pico kit Affymetrix RNA amplification kit (Nugene) according to manufacturer's protocol. Microarray analysis of mouse EpCAM-positive cells was performed using Affymetrix Clariom® S 8-Array HT Plate according to the standard protocols of the Dutch Genomics Service and Support Provider (MAD, Science Park, University of Amsterdam, Netherlands). The data was normalised using Expression Console 1.4.1.46 and uploaded to R2: Genomics Analysis and Visualization Platform (http://hgserver1.amc.nl/). Microarray results were analysed using R2 software. Differentially expressed genes were selected based on fold change (≥ 2) in comparison to control group. For gene set enrichment analysis, sets of genes from the Molecular Signatures database (MSigDB, http://software.broadinstitute.org/gsea/msigdb/genesets.jsp) and the GEO GSE92332 [88] data set were extracted and signatures of HALLMARK Vacuole organization, GO Fatty acid metabolism, proximal and distal enterocytes, and proximal and distal enteroendocrine cells were compared to our data set using GSEA software (http://software.broadinstitute.org/gsea/index.jsp).

Seahorse analysis of fetal organoids

Fetal organoids were treated with antibiotics described above starting from day 1 of culture and transferred to XFe24 cell culture microplate (Agilent) at day 3 of culture, with new medium containing the same antibiotics. After 2 days, organoid wells were washed twice with PBS, assay medium (Agilent) was added and organoids were kept without CO_2 till the start of the measurements. Oxygen consumption rate and extracellular acidification rate were measured in an XF24 seahorse machine (Agilent) according to the manufacturer's instructions. Immediately after, organoids were collected from the matrigel using Cell Recovery Solution (Corning B.V.) and DNA was extracted and measured in nanodrop to normalize experimental values.

Assay medium: DMEM (Sigma D5030) with 4 mM glutamine and, for OCR assay, with 10 mM glucose.

Concentration of injected compounds: 1 µM oligomycin, 0.5 µM FCCP, 1 µM rotenone, 1 µM antimycin A, 10 mM glucose, and 50 mM 2-DG.

Compounds used to challenge mitochondrial respiration: oligomycin represses ATP production in the mitochondria, decreasing OCR; carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) stimulates respiration to the maximum capacity of the mitochondria, increasing OCR; Rotenone and Antimycin A (Rot + Ant) completely shuts down respiration, decreasing OCR.

Compounds used to challenge glycolysis: glucose is added to the glucose-depleted medium, increasing ECAR to basal conditions, oligomycin shifts ATP production from mitochondrial respiration to glycolysis, increasing ECAR; 2-deoxy-glucose (2-DG) inhibits glycolysis, decreasing ECAR.

Basal respiration was calculated by subtracting the minimum rate after adding rotenone and antimycin A from the last rate measurement before adding oligomycin. ATP production was calculated by subtracting the minimum rate measurement after adding oligomycin from the last rate measurement before adding oligomycin. Maximal Respiration was calculated by subtracting the minimum rate after adding rotenone and antimycin A from the maximum rate measurement after adding FCCP.

Glycolysis was calculated by subtracting the last rate measurement before adding glucose from the maximum rate measurement before adding oligomycin. Glycolytic capacity was calculated by subtracting the last rate measurement before adding glucose from the maximum rate measurement after adding oligomycin.

Software

nQuery 7.0: sample-size calculations
ImageJ: villi length and crypt depth measurement; SIS immunostaining quantification
Transcriptome Analysis Controle (TAC): microarray data analysis, PCA and volcano plots creation and identification of 2-fold differentially expressed genes
R2: heatmaps creation
GSEA software (http://software.broadinstitute.org/gsea/index.jsp): GSEA analysis
LinRegPCR: quantitative Real-time PCR analysis
GeNorm: identification of most stable reference genes for quantitative Real-time PCR analysis
GraphPad Prism 8: statistical analysis and graph creation

Statistical Analysis

Sample size was calculated using nQuery and based on maturation studies, using a two-group t-test of equal n's, with a significance level (α) of 0,05 and power of 80%. There were no exclusions or drop-outs to report. Analysis was performed with blinded data. Data were analyzed using GraphPad Prism 8 and are presented as mean ± Standard Deviation unless stated otherwise in the figure legends. Sample distribution was determined using D'Agostino and Pearson normality test. Sample numbers, experimental replicates, type of statistical analysis test, and p values are reported in the figure legends. 2-way ANOVA was used for time-course experiments of non-repetitive measurements, as differences at each time point were analyzed.

Data availability

Microarray data are deposited in the Gene Expression Omnibus Database (GSE172061).

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5

Altered Gut Anti-Bacterial Defense In Adult Mice Treated With Antibiotics During Early Life

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Antibiotics, 2022, 11(2): 267

ABSTRACT

The association between prolonged antibiotic (AB) use in neonates and increased incidence of later life diseases is not yet fully understood. AB treatment in early life alters intestinal epithelial cell composition, functioning and maturation, which could be the basis for later life health effects. Here, we investigated whether AB-induced changes in the neonatal gut persisted up to adulthood and whether early life AB had additional long-term consequences for gut functioning. Mice received AB orally from postnatal day 10 to 20. Intestinal morphology, permeability, and gene and protein expression at 8 weeks were analyzed. Our data showed that the majority of the early life AB-induced gut effects did not persist into adulthood, yet early life AB did impact later life gut functioning. Specifically, the proximal small intestine (SI) of adult mice treated with AB in early life was characterized by hyperproliferative crypts, increased number of Paneth cells, and alterations in enteroendocrine cell-specific gene expression profiles. The distal SI of adult mice, displayed a reduced expression of antibacterial defense markers. Together, our results suggest that early life AB lead to structural and physiological changes in the adult gut, which may contribute to disease development when homeostatic conditions are under challenge.

INTRODUCTION

Early life is a crucial moment in the development of mammals. Events that occur during the first weeks or months after birth can imprint long-lasting effects [1-4]. Among the external factors that can affect long-term health are early life antibiotics (AB). AB are commonly prescribed to neonates and children [5-7] due to the high incidence of respiratory infections in pediatric patients [8]. Besides, early life AB are used to treat gastrointestinal, skin, and ear infections. Many neonates also receive AB because of suspected early onset sepsis, especially after preterm birth [9]. However, in most cases bacterial infection is not culture-proven in uninfected infants [10]. Especially in the case of premature neonates, AB are used to treat sepsis or to prevent infection when newborn or maternal risk factors are present. Among the different classes of AB, the most used during early life are β-lactams (amoxicillin, ampicillin), aminoglycosides (gentamycin, amikacin), glycopeptides (vancomycin, teicoplanin), and nitroimidazoles (metronidazole).

In recent years, prolonged treatment with early life AB in humans has been linked with several pathologies later in life, such as obesity [11-14], diabetes [15,16], and inflammatory bowel diseases (IBD) [17-19]. As the gut is home to trillions of commensal bacteria, essential to the balanced functioning of an organism, it is not surprising that the disruption of the microbiome by AB can lead to gut-related pathologies. In preclinical mouse models, it has been shown that AB treatment in early life leads to changes in both gut microbiome and intestinal immunity that persist into adulthood, long after the treatment has stopped [20,21]. Moreover, the maturation of the intestinal epithelium is only completed after birth and alterations during this vulnerable period can disturb the normal maturation process and might lead to long-term consequences. We have recently demonstrated that antibiotic treatment during early life can directly affect the intestinal epithelial cell (IEC) composition and functioning, independent of the effects on the microbiome [22]. Pups treated with AB between P10 and P20 showed accelerated intestinal maturation, as demonstrated by the decrease in intestinal permeability and the disappearance of specialized vacuolated cells, characteristic of the neonatal period. Furthermore, in vivo AB treatment of neonatal pups and in vitro AB treatment of fetal intestinal organoids induced the expression of adult specific brush-border enzymes, in both proximal and distal SI, and enteroendocrine cell (EEC) markers, particularly in the proximal SI [22]. Finally, AB-treated pups displayed higher expression of innate defense markers, especially in the distal SI [22]. Previous studies have also shown that adult mice that received AB during weaning are more susceptible to intestinal colitis and colon cancer when challenged with DSS and/or azoxymethane [23-26]. Altogether, these data suggest that the direct and indirect effects of AB on the immature neonatal gut can have

long-lasting consequences and predispose to gut related diseases. Yet, the long-term effects of early life AB observed in the homeostatic gut, i.e., without any challenge, have never been described. Therefore, it is essential to identify the direct impact of early life AB on gut development and later life gut functioning in order to better target gut health and prevent diseases.

In the current study, we investigated whether the epithelial changes resulting from the direct effect of AB early in life, i.e., before and during weaning, persist in the adult intestine, at 8 weeks of age. Furthermore, by analyzing the global expression of adult IECs we identified additional long-term consequences of AB in early life.

RESULTS

Adult mice treated with early life antibiotics show altered small intestine morphology

To investigate the long-term effects of early life AB, we treated mice daily by oral gavage, from postnatal day (P)10 to P20, with a mix of AB comprising the most commonly used classes in neonates: amoxicillin, vancomycin, and metronidazole (Figure 1A). No aminoglycoside AB was used as this class is not absorbed by the gastrointestinal epithelial cells. At P20, AB treatment ended and mice were weaned (Figure 1A). As of week 3, bodyweight was similar between AB-treated mice and control mice, which received PBS (Figure 1B). We previously showed that the intestinal permeability of P20 pups treated with the same early life AB mix was reduced compared to control pups [22]. To evaluate whether this difference in intestinal permeability persists into adulthood, we orally administered FITC-dextran to 8-week-old mice, that had been treated with AB in early life, and quantified its concentration in serum four hours later (Figure 1C). FITC-dextran serum concentration was identical in AB-treated and control mice indicating that intestinal permeability normalized over time after AB treatment (Figure 1D). Still, analysis of the small intestine (SI) showed that both its weight and length were significantly higher in mice that received early life AB compared to control mice (Figure 1E and 1F). Finally, the liver weight of AB-treated mice was significantly lower than control mice, while spleen weight was not different between both groups (Figure 1G and 1H). Although the differences in intestinal permeability immediately after AB treatment are no longer present in adulthood, the increased weight and length of the SI suggest that early life AB have long-lasting effects on the intestine.

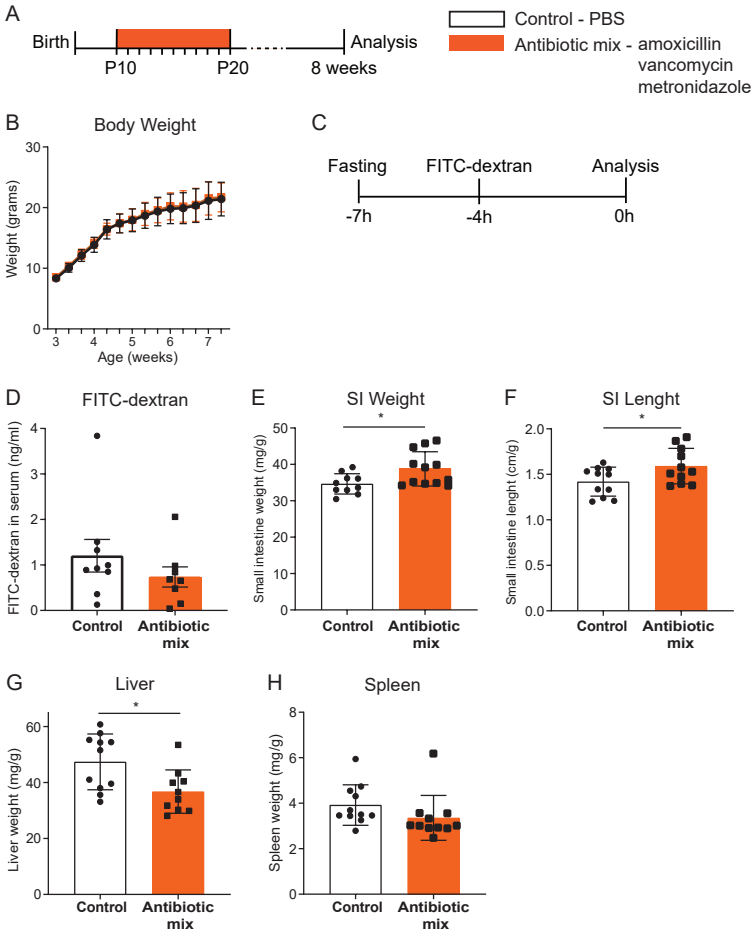

Figure 1. Growth, intestinal permeability, and macroscopic assessment of small intestine, liver, and spleen.

(A) Experimental design of in vivo antibiotic treatment of pups between postnatal (P) day 10 and P20. Antibiotic mix: amoxicillin, metronidazole, and vancomycin. All analyses were done at 8 weeks of age. (B) Mice weight was measured three times per week after antibiotic mix treatment, n = 11 control mice, n = 12 AB-treated mice. (C-D) Permeability assay assessed by FITC-dextran concentration in serum 4 hours after oral gavage, n = 9 control mice, n = 8 AB-treated mice. (E) Small intestine weight, relative to body weight, n = 10 control mice, n = 12 AB-treated mice. (H) Small intestine length, relative to body weight, n = 11 control mice, n = 11 AB-treated mice. (F) Liver weight, relative to body weight, n = 11 control mice, n = 10 AB-treated mice. (J) Spleen weight, relative to body weight, n = 11 control mice, n = 11 AB-treated mice. Statistical analysis was performed by two-way ANOVA test with Sidak's multiple comparisons test (B) or two-tailed unpaired t-test (C-J). Error bars indicate mean ± SD. Levels of significance are indicated (*p<0.05).

Early life antibiotics induce hyperproliferative crypts in adult proximal small intestine

The epithelial cells of the proximal and distal SI have different genetic profiles that support specific regional nutrient absorption and digestion functions, as well as different immune defense requirements [27-30]. We previously showed that these two SI

regions are differently affected by early life AB treatment at P20 [22], thus in the present study we analyzed adult proximal and distal SI separately. Assessment of SI histology revealed no major morphological differences (Figure 2A). A trend towards shorter villi and significantly longer crypts was observed in the proximal SI of AB-treated mice compared to control mice, while distal SI showed similar villus lengths and crypt depths

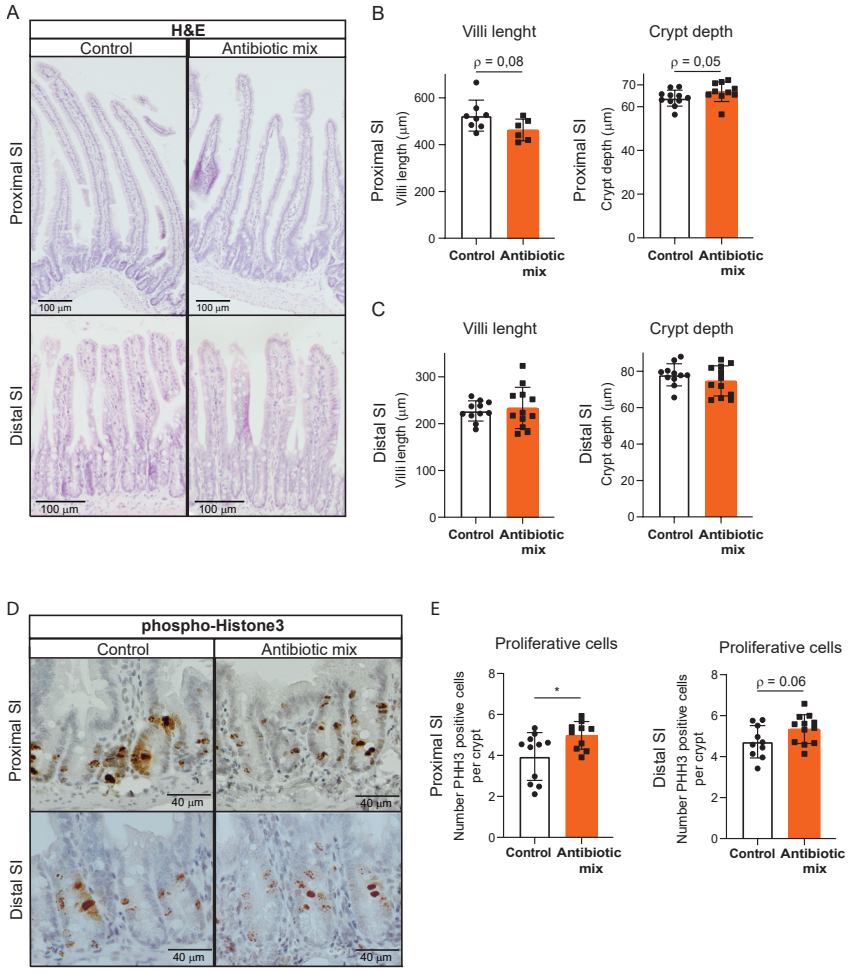

Figure 2. Hyperpoliferative crypts in adult mice treated with early life antibiotics.
(A) H&E staining of proximal and distal small intestine. (Scale bars, 100 µm.) (B) Villus length and crypt depth in proximal small intestine. Villus: n = 8 control mice, n = 6 AB-treated mice; crypt: n = 11 control mice, n = 10 AB-treated mice. (C) Villus length and crypt depth in distal small intestine. Villus: n = 11 control mice, n = 12 AB-treated mice; crypt: n = 11 control mice, n = 12 AB-treated mice. (D and E) Immunohistochemistry of proliferation marker phosphorylated histone H3 (D) and quantifications of positive stained cells (E) in proximal and distal small intestine. Proximal: n = 11 control mice, n = 10 AB-treated mice; distal: n = 10 control mice, n = 12 AB-treated mice. (Scale bars, 40 µm.) Statistical analysis was performed by Mann-Whitney test as data was not normally distributed when assessed by D'Agostino and Pearson normality test (B) or two-tailed unpaired t-test (C and E). Error bars indicate median with interquartile range (B) or mean ± SD (C and E). Levels of significance are indicated (*p<0.05).

in the two groups (Figure 2B and 2C). As longer crypts suggest increased proliferation, we performed staining for the mitotic marker phosphorylated histone H3 (PHH3) (Figure 2D). Proximal SI of AB-treated mice displayed a higher number of PHHH3 stained cells compared to control mice and in the distal SI of these mice only a trend toward increased PHHH3 stained cells was noted (Figure 2E). These results indicate that early life AB induce hyperproliferative crypts, especially in the proximal SI.

The majority of the direct effects induced by antibiotics on the neonatal small intestine do not persist into adulthood

The postnatal maturation of the mouse intestinal epithelium is characterized by several changes in its morphology and function. For example, from P14 to P28, the SI brush-border starts to express adult specific enzymes, while the number of Paneth cells expands in the newly developed crypts [31]. We have shown that AB treatment of mice between P10 and P20 led to upregulation of the adult brush-border enzymes and Paneth cells at P20 [22]. We also demonstrated that these changes resulted of the direct effect of AB on IECs [22]. Thus, here we set out to determine whether these effects were still present in adult mice that received the same AB treatment in early life. In contrast with the observations at P20, whole tissue qRT-PCR analysis on the adult proximal SI showed similar relative expression of the brush-border enzymes Sis and Arg2 between AB-treated mice and control adult mice (Figure 3A). However, the relative expression of the Paneth cell marker lysozyme-1 (Lyz1) was higher in AB-treated adult mice compared to control mice (Figure 3A). Immunohistochemistry of Lyz1 also revealed a significantly higher number of Paneth cells in the adult proximal SI after early life AB treatment (Figure 3B). In the distal SI, Arg2 showed a trend towards an increased relative expression after AB treatment, but Sis and Lyz1 were similarly expressed between the two groups (Figure 3C). In accordance, immunohistochemistry of Lyz1 showed similar amount of Paneth cells in the distal SI in both groups (Figure 3D).

The upregulation of EEC markers observed after AB treatment in proximal SI at P20 did not persist into adulthood, as measured by qRT-PCR of 8-week-old SI whole tissue (Figure 3E). Only the relative expression of secretin (Sct) remained significantly higher, while cholecystokinin (Cck), which relative expression was not changed by AB at P20, was significantly lower in AB-treated adult mice compared to control mice (Figure 3E).

Overall, the adult SI recovers from the direct effects of early life AB treatment, with the exception of the greater number of Paneth cells, the higher relative expression of Sct, and the lower relative expression of Cck in the proximal SI.

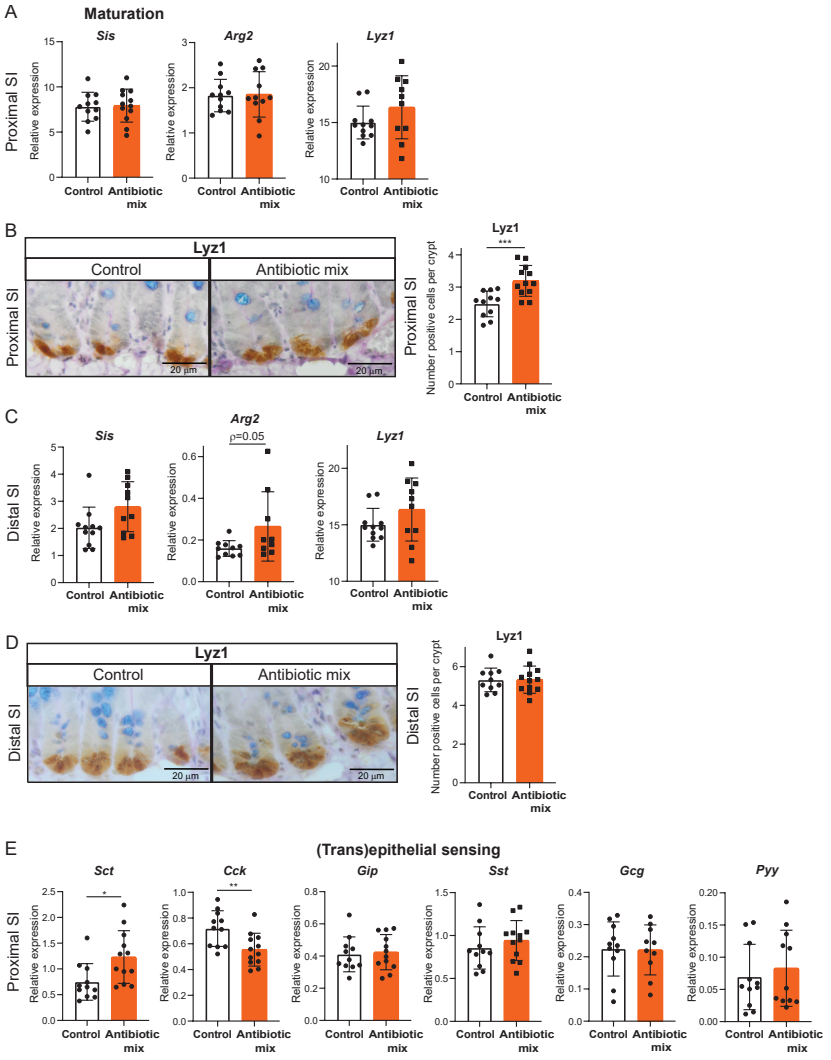

Figure 3. Antibiotics in early life lead to higher number of Paneth cells and few changes in enteroendocrine markers in the proximal small intestine.

(A) Whole tissue Real-time qPCR analysis of adult brush-border enzymes Sis and Arg2 and of Paneth cell marker Lyz1 in proximal small intestine. Relative expression to reference genes Rpl4 and Ppib. Sis: n = 11 control mice, n = 12 AB-treated mice; Arg2: n = 11 control mice, n = 11 AB-treated mice; Lyz1: n = 11 control mice, n = 10 AB-treated mice. (B) Immunohistochemistry of lysozyme-1 in proximal small intestine, n = 11 control mice, n = 12 AB-treated mice. (Scale bars, 20 µm.) (C) Whole tissue Real-time qPCR analysis of adult brush-border enzymes Sis and Arg2 and of Paneth cell marker Lyz1 in distal small intestine. Relative expression to reference genes Rpl4 and Ppib. Sis: n = 11 control mice, n = 10 AB-treated mice; Arg2: n = 10 control mice, n = 9 AB-treated mice; Lyz1: n = 11 control mice, n = 10 AB-treated mice. (D) Immunohistochemistry of lysozyme-1 in distal small intestine, n = 10 control mice, n = 12 AB-treated mice (Scale bars, 20 µm.) (E) Whole tissue Real-time qPCR analysis of enteroendocrine markers Sct, Cck, Gip, Sst, Gcg, and Pyy in proximal small intestine. Relative expression to reference genes Rpl4 and Ppib. Sct, Cck, Gip, Sst: n = 11 control mice, n = 12 AB-treated mice. Gcg: n = 11 control mice, n = 10 AB-treated mice. Pyy: n = 11 control mice, n = 11 AB-treated mice. Statistical analysis was performed by one-tailed unpaired t-test or Mann-Whitney test, as data was not normally distributed when assessed by D'Agostino and Pearson normality test (Sct). Error bars indicate mean ± SD or median with interquartile range (Sct). Levels of significance are indicated (*p<0.05, **p<0.01).

Genome-wide gene expression analysis reveals modest differences in epithelial cells of adult mice treated with antibiotics in early life

Early life AB treatment broadly depletes the commensal microbiota of the developing gut, which affects the IECs gene expression profile in early life [13,20,23,24,32]. The unbalanced microbiota expansion that follows after the AB treatment at P20 can also affect IEC-specific gene expression. To examine whether the AB-induced changes in IEC gene expression persist into adulthood, we next performed genome-wide gene expression analysis on mRNA from proximal and distal SI epithelial cells of 8-week-old mice that had received AB or PBS in early life (Figure 4A). Principal component analysis (PCA) showed a modest separation between AB-treated and control proximal SI epithelial cells along the second component (PC2 23.1%) (Figure 4B). This separation was not found in the analysis of distal SI epithelial cells (Figure 4C). Nevertheless, differential gene expression analysis showed a similar number of at least 2-fold upregulated or downregulated genes in AB-treated epithelial cells from both regions (Figure 4D and 4E). Specifically, 92 genes in proximal SI and 137 genes in distal SI epithelial cells were upregulated. Next, we grouped these differently expressed genes into specific functions. This resulted in a single function being clearly affected specifically in the distal SI: “antibacterial defense”. These data showed that, of the few differences in adult IECs gene expression caused by AB treatment in early life, “antibacterial defense” seems to be the only function affected especially in the distal SI.

Intestinal antibacterial defense is reduced in distal small intestine of adult mice that received antibiotics in early life

The identified genes involved in antibacterial defense were downregulated in the distal SI epithelial cells of AB-treated mice compared to control mice at 8 weeks of age (Figure 5A). This is in contrast with our findings at P20 which showed upregulated innate defense markers, including Reg3 lectins, in AB-treated pups [22]. Thus, we then compared the gene expression of these antibacterial defense genes between the distal SI of P20 pups and adult mice. We found that while control adult mice showed a sharp increase in the expression of antibacterial defense genes compared to control neonatal pups (Figure 5B), AB-treated adult mice showed similar expression levels of these genes compared to AB-treated neonatal pups (Figure 5C). This indicates that the developmental upregulation in gene expression of antibacterial defense markers taking place in the distal SI of control mice does not occur in AB-treated mice.

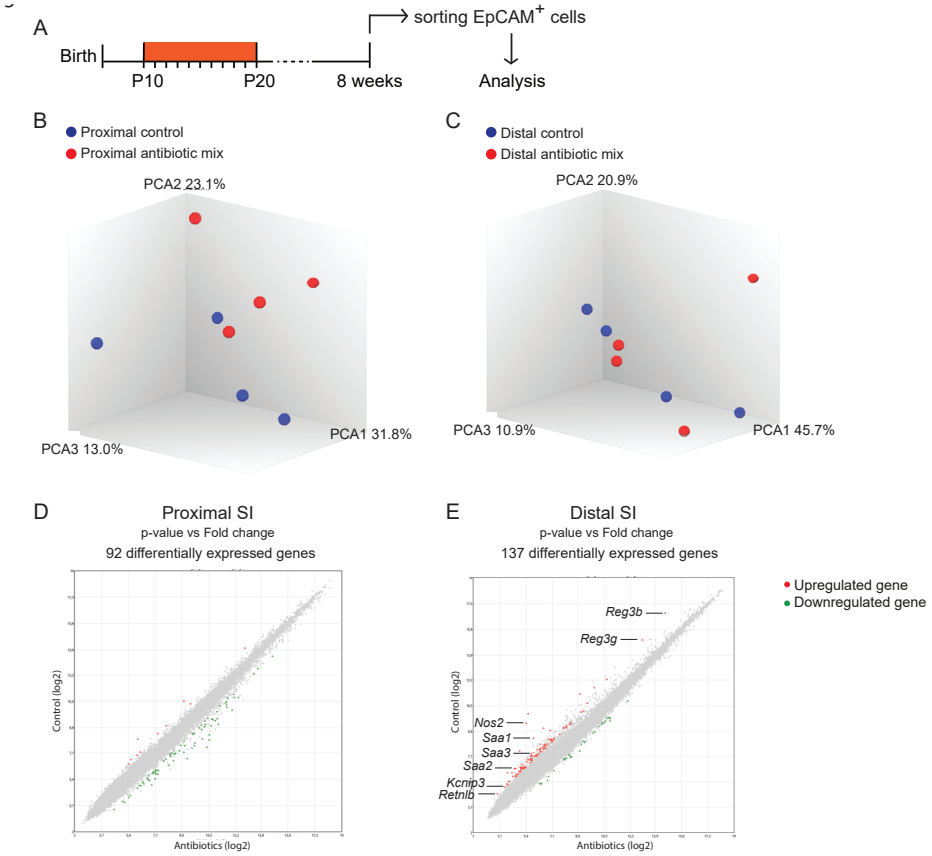

Figure 4. Genome-wide gene expression analysis of sorted intestinal epithelial cells.
(A) Experimental design of genome-wide gene expression analysis of FACS-sorted intestinal epithelial cells of 8 week old mice. (B-C) PCA analysis of sorted epithelial cells from 8 week old proximal (B) and distal (C) small intestine treated with the antibiotic mix or PBS (control) in early life. (D-E) Volcano plots of microarray analysis showing genes differentially expressed between control and antibiotic-treated FACS-sorted intestinal epithelial cells of proximal (D) and distal (E) small intestine of 8 week old mice. Green dots identify downregulated genes and red dots identify upregulated genes. Statistical analysis by ANOVA eBayes, $p < 0.05$ cut-off, $n = 4$ samples per group.

Colitis is one of the pathologies that has been associated with the use of antibiotics during early life [17-19]. We examined in more detail the expression of regenerating islet-derived protein 3 lectins (Reg3) and serum amyloid A proteins (Saa), as these have been described to be inversely correlated with the development of colitis [33,34]. An independent experiment was performed with exactly the same conditions and whole distal SI was analyzed by qRT-PCR, confirming these results, except for Saa3 (Figure 5D). Finally, visualization of goblet cells, which are key players in gut barrier defense, by staining of mucins, showed a similar number of this secretory cell type between antibiotic-treated and control mice (Figure 5E). Overall, these results showed that early life AB treatment limits intestinal antibacterial defense in the distal small intestine by preventing the normal developmental pattern of these defense genes from weaning into adulthood.

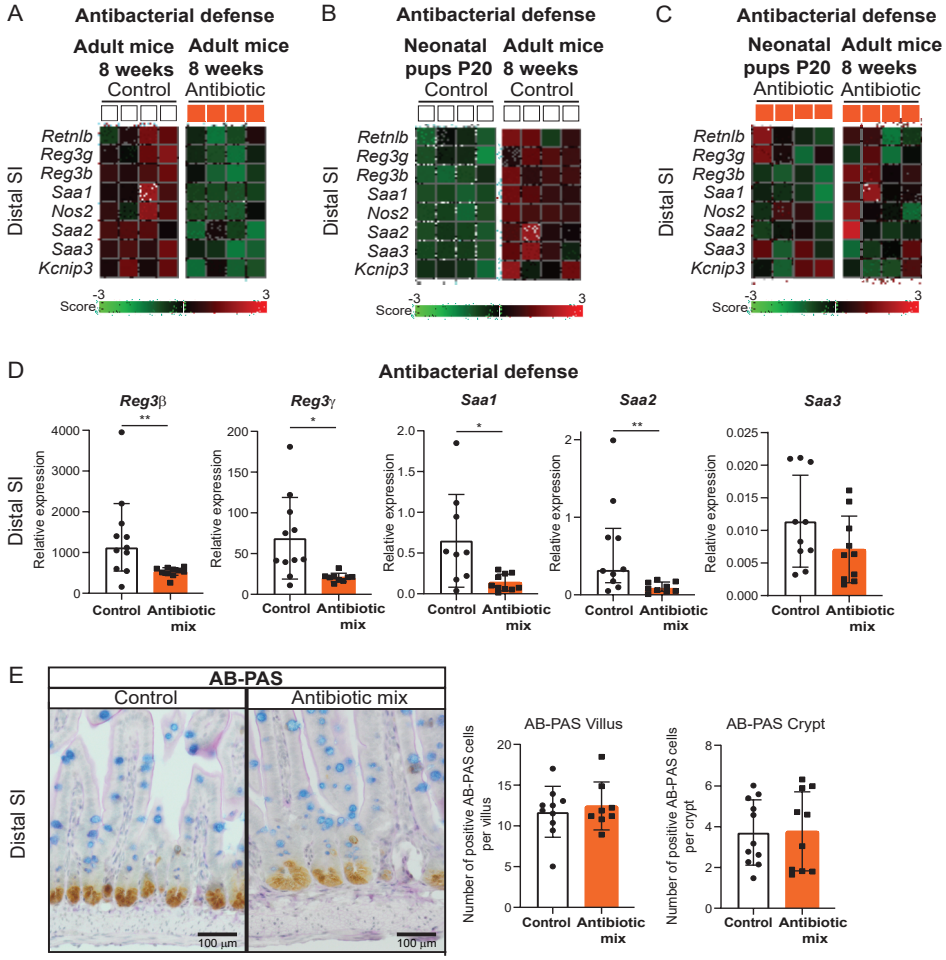

Figure 5. Reduced antibacterial defense in distal small intestine of adult mice treated with early life antibiotics. Differential gene expression analysis of sorted intestinal epithelial cells after antibiotic treatment.

(A-C) Curated heatmaps of selected genes from top downregulated and top upregulated genes, based on biological interest and grouped according to function "antibacterial defense" in distal epithelial cells of control and AB-treated adult mice (A), control neonatal pups and adult mice (B), and AB-treated neonatal pups and adult mice (C). The colored bar represents the expression level from low (green) to high (red), n = 4 samples per group. (D) Whole tissue Real-time qPCR analysis of antibacterial defense markers Reg3β, Reg3γ, Saa1, Saa2, and Saa3 in distal small intestine. Relative expression to reference genes Rpl4 and Ppib. Reg3β: n = 11 control mice, n = 10 AB-treated mice. Reg3γ: n = 11 control mice, n = 10 AB-treated mice. Saa1: n = 9 control mice, n = 10 AB-treated mice. Saa2: n = 10 control mice, n = 9 AB-treated mice. Saa3: n = 10 control mice, n = 10 AB-treated mice. (E) Staining and quantification of mucins in villus and crypts of distal small intestine. Villi: n = 10 control mice, n = 8 AB-treated mice; crypts: n = 11 control mice, n = 10 AB-treated mice. (Scale bars, 100 µm.) Statistical analysis by ANOVA eBayes, $p < 0.05$ cut-off (A-C) or by one-tailed unpaired t-test or Mann-Whitney test (Reg3β, Saa2), as data was not normally distributed when assessed by D'Agostino and Pearson normality test (Sct). Error bars indicate mean ± SD or median with interquartile range (Reg3β, Saa2). Levels of significance are indicated (*p<0.05, **p<0.01).

DISCUSSION

In this study, we found that pre-weaning treatment of young mice with AB results in permanent higher SI weight and increased SI length later in life (Figure 1). This might be partly explained by the crypt hyperproliferation observed in the proximal SI of AB-treated mice, supported by the higher amount of Paneth cells, and the consequent increase in crypt depth (Figure 2 and 3). Certain bacterial strains can influence crypt proliferation. Specifically, gram-positive bacteria and associated short-chain fatty acids (SCFAs) production are known to increase IECs turnover [35]. Lactic-acid-producing bacteria have also been shown to stimulate intestinal stem cell proliferation [36]. Examination of the intestinal bacterial composition before and after AB treatment, as well as later in life, could provide additional insights to whether the observed increase in SI weight, length and proliferation is due to microbial signals. Nevertheless, the fact that morphological differences are only evident in the poorly microbial-populated proximal SI compared to the distal SI indicates that these differences might be independent of microbiota.

The strongly reduced intestinal permeability measured at P20 [22] was not observed in adulthood (Figure 1). Decreased permeability, and thus increased barrier function, is one of the hallmarks of intestinal epithelial maturation that in mice occurs between the second and fourth week after birth [37]. The similarity in intestinal permeability observed in adulthood between AB-treated and control mice further demonstrates that its reduction at P20 was a result of the accelerated maturation caused by AB treatment and, thus, an early life effect.

Although the increase in expression of adult brush-border genes was a short-term effect of early life AB in both SI regions [22], at 8 weeks only Arg2 remained increased in the distal SI of AB-treated mice (Figure 3). Bacteria have been linked to Arg2 expression [38,39], which could explain its specific increase in distal SI at 8 weeks of age. Previous studies have demonstrated that altered microbiota composition induced by AB during early life can persist into adulthood [12,20,24,40]. Therefore, it may well be that the microbiota of 8-week-old AB-treated mice is associated with changes in epithelial expression, as we observed for Arg2.

At P20, early life AB treatment induced expression of EEC markers in the proximal SI of both neonatal pups and fetal intestinal organoids, demonstrating that the increase in EEC gene expression and number of EEC is a direct effect of AB on IECs [22]. In AB-treated adult mice, only the expression of Sct remained upregulated, while Cck gene expression, which was not changed at P20, was downregulated (Figure 3). EEC secrete hormones that regulate digestion, epithelial barrier integrity, and mucosal immunity [41]. Innap-

propriate release of EEC hormones can be involved in the development of obesity and diabetes [42-44]. For example, the marker Sct encodes the hormone secretin which induces insulin release. When insulin levels are persistently high, cells develop insulin resistance, an early sign of diabetes type2. In our study, we observed a higher relative gene expression of Sct in AB-treated mice, which could contribute to AB-linked development of diabetes 2 [45,46]. However, it is not known whether the increase in Sct relative expression causes continuous increased insulin release and consequently diabetes type 2 development. Also the downregulated Cck expression in AB-treated adult mice might have detrimental effects on bacterial growth and intestinal inflammatory conditions. Specifically, decreased Cck might limit the capacity of the gut to prevent bacterial overgrowth and lead to increased translocation of bacteria into the intestinal mucosa [47], which can stimulate pro-inflammatory conditions and exarcebate intestinal inflammation.

Antibacterial defense in the distal SI was the only function still profoundly affected at 8 weeks of age (Figure 5). The expression of antibacterial genes increases from the neonatal stage to adulthood, but AB blocked this increase. The distal SI epithelium is not capable of recovering from the AB treatment and the antibacterial defense gene expression levels in control adult mice are not reached in AB adult mice. Thus, not only the negative impact on the gut microbiome and intestinal immunity caused by early life AB persists in adulthood as described previously [20,21] but also epithelial defense response is affected into adulthood. Consequently, the distal SI of adult mice that received early life AB is less protected at homeostatic conditions, which will leave it more vulnerable to bacterial infections and dysbiosis, thereby increasing the susceptibility to disease. Besides, Reg3 lectins, which were among the downregulated antibacterial defense markers, have bactericidal activity against gram-positive bacteria, controlling their presence in the gut and contributing to the microbiome composition [48-51] and lower Reg3 expression is also associated with a higher risk of colitis in mice [34]. Intestinal chronic inflammation pathologies, such as ulcerative colitis and Crohn's disease, result from inadequate response to intestinal microbiota, including commensal bacteria. Decreased intestinal antibacterial defense due to early life AB treatment can lead to dysbiosis, promoting the onset of inflammatory conditions. Ultimately, bacteria may more easily translocate from the lumen to extraintestinal sites and contribute to the exacerbation of inflammation symptoms. Indeed, adult mice that received AB in early life showed increased susceptibility to DSS-induced colitis, colon cancer, obesity, and diabetes [11,16,23,40]. In addition, a recent systematic review of clinical studies in children showed strong evidence for an association between early life AB and both IBD and celiac disease in later childhood [52]. IBD is a multifactorial disease affecting the gut immune system, epithelium, and microbiome. Previously it was demonstrated that

early life AB causes changes in the microbiome that persist into adulthood, accompanied by a negative impact on the immune system [20,21]. Our present study shows that the epithelial defense response is yet another aspect of the long-lasting effects of AB in early life. Further studies are required to demonstrate the association between reduced epithelial antibacterial defense and increased predisposition to IBD, and to understand the underlying mechanisms.

In conclusion, AB in early life leads in adulthood to intestinal structural differences, hyperproliferation of the proximal SI, increased Sct and decreased Cck expression in the proximal SI, and impaired antibacterial defense in the distal SI, which may contribute to the increased incidence of gut-related diseases when homeostatic conditions are challenged. Validation of these findings in the human small intestine will help to develop targeted and personalized strategies, for neonates and children that receive prolonged antibiotic treatment, for example using prebiotics, probiotics, or synbiotics.

MATERIALS AND METHODS

In vivo studies

This study was conducted in accordance with institutional guidelines for the care and use of laboratory animals established by the Animal Ethics Committee of the University of Amsterdam, and all animal procedures related to the purpose of the research were approved under the Ethical license of the national competent authority, securing full compliance the European Directive 2010/63/EU for the use of animals for scientific purposes.

Six pregnant 8 weeks old C57Bl/6J females were obtained from Charles River and were allowed to adapt to the new environment for 1 week. Pregnant females were individually housed and received AIN-93G diet (Triple A Trading/Altromin). Mice were kept in innovive disposable mice cages, enriched with corncob bedding and carton house, with tissue as nesting material, under a strict 7am to 7pm dark-light cycle, controlled temperature and humidity, with food and water given *ad libitum*. Pups were monitored daily, weighted every other day from P10 onwards and kept with the mothers throughout the experiment. At P10, two experimental groups were randomly defined: treatment group (3 litters, 4 pups per litter) received daily oral gavage of 30µl of AB (25mg/kg/day amoxicillin (Amsterdam UMC, AMC pharmacy), 50mg/kg/day metronidazole (Amsterdam UMC, AMC pharmacy) and 50mg/kg/day vancomycin (Sigma/Aldrich)); control group (3 litters, 4 pups per litter) received daily oral gavage of 30µl of PBS. AB or PBS were consistently given during the light period, always at the same time period of the day, nonblinded. For

oral gavage, pups were separated from the mother all at once and placed back all at once as well, to correct for differences in maternal care. On P21, pups were weaned and the 6 litters were distributed into 4 different cages: control females, control males, antibiotic-treated females, and antibiotic-treated males. Mice were monitored daily and weighted every other day. At 8 weeks, mice were fasted and after 3 hours, 250µl of 60mg/100gr weight FITC-dextran 4kDa (Sigma/Aldrich) diluted in PBS were given via oral gavage to all the mice, to assess intestinal permeability. After 4 hours, mice were euthanized by CO_2 and isoflurane exposure. Immediately after, blood was collected by heart puncture in MiniCollect® Z Serum Sep Clot tubes (Greiner). After 30 minutes incubation on ice, in the dark, blood was centrifuged and serum was collected and kept at -80°C.

FITC-dextran *in vivo* permeability assay

Standard samples were obtained by 2-fold serial dilution of 1mg/ml FITC-dextran in blood serum. The fluorescence signals of the serum samples were recorded with an excitation wavelength of 485 nm and emission wavelength of 520 nm and compared with the standard curve values. The amount of FITC-dextran in serum samples was calculated in ng/ml.

Immunostaining

Tissue was flushed with PBS, fixed overnight in 4% formaldehyde, embedded in paraffin and sectioned. Sections were deparaffinised with xylene and gradually rehydrated in ethanol. After blocking the endogenous peroxidase (0,01% H2O2 in methanol), slides were boiled in 0,01M sodium citrate buffer (pH 6) for 10 minutes at 120˚C in a autoclave for antigen retrieval. Slides were blocked for 30 minutes at room temperature in PBS with 1% bovine serum albumin and 0,1% Triton-X-100. Then, slides were incubated overnight with primary antibody diluted in the blocking buffer. Slides were washed with PBS and secondary antibody diluted in blocking buffer was added for 30 minutes at room temperature. Antibody binding was visualized by adding chromagene substrate diaminobenzedine (Sigma-Aldrich), sections were counterstained using haematoxillin (Sigma) and slides were dehydrated and mounted with entellan.

For alcian blue and periodic acid (AB-PAS) staining, deparaffinised sections were stained with alcian blue (Sigma, A3157) for 20 minutes at room temperature, washed under running tap water for 5 minutes and then in bidistilled water before incubating with freshly prepared 0.1 % periodic acid (Sigma, P0430). Slides were then washed once more under running tap water for 10 minutes and stained with Schiff's reagent (VwR, J62171.AP) also for 10 minutes. Slides were washed under running tap water for 5 minutes and in bidistilled water for another 5 minutes. Finally, sections were counterstained with haematoxillin (Sigma) and slides were dehydrated and mounted with entellan. Sections

were examined using brightfield microscope Olympus BX51 and analysis was performed with blinded slides. Per mouse, the length of at least 10 villi and the depth of at least 10 crypts were measured. For AB-PAS analysis, at least 20 villi and 40 crypts were quantified. To quantify the number of phosphor-histone H3 and LYZ1 stained cells per crypt, at least 35 crypts in the proximal SI and at least 40 crypts in the distal SI were quantified, per mouse.

Phospho-Histone H3 – rabbit polyclonal anti-phospho-histone H3 (1:200, ThermoFischer, PA5-17869)
LYZ1 – rabbit polyclonal anti-lysozyme (1:2000, DAKO, A0099)

Epithelial cells FACS-sorting

Small intestine of 8-weeks-old mice was cut open and proximal and distal parts were separated, cut into pieces and washed with ice-cold PBS. Crypts were dissociated after incubation with 2 mM EDTA (Merck/VWR) for 30 minutes at 4°C and filtered through a 70 µm cell strainer (BD/VWR). Single cells were obtained by incubating crypts with TrypLE Express (Invitrogen). Cells were kept in PBS 2% FCS Rho-kinase inhibitor and RNase inhibitor (Fermentas/Thermo Fisher Scientific) solution and stained with EpCAM-FITC antibody (1:50, BioLegend, 324204) for 30 minutes on ice.

RNA isolation and qRT-PCR

For transcriptome profiling, RNA was extracted from EpCAM-positive cells using phenol-chloroform method. RNA quality was measured on an Agilent 2100 Bioanalyzer.

For qRT-PCR, RNA from whole-tissue tissue was isolated using the Bioline ISOLATE II RNA Mini kit (BIO-52073, Bioline) according to manufacturers' instructions. 1 µg of RNA was transcribed using Revertaid reverse transcriptase according to protocol (Fermentas, Vilnius, Lithuania). Quantitative RT-PCR was performed on a BioRad iCycler using sensifast SYBR No-ROX Kit (GC-biotech Bio-98020) according to manufacturer's protocol. The 2 most stable reference genes were determined using GeNorm and their geometric mean was used to calculate relative expression of genes of interest: for whole-tissue qRT-PCR ribosomal protein L4 (*Rpl4*) and peptidylpropyl isomerase B (*Ppib*). Relative gene expression was calculated using N0 values obtained by LinRegPCR analysis. Primers were previously validated using melting curve analyses and gel electrophoresis of PCR product.

Rpl4: FW – CCTTCTCCTCTCCCCGTCA ; RV - GCATAGGGCTGTCTGTTGTTT
Ppib: FW – GCCAACGATAAGAAGAAGGGA; RV - TCCAAAGAGTCCAAAGACGAC
Sis: FW – TGCCTGCTGTGGAAGAAGTAA; RV - CAGCCACGCTCTTCACATTT

Arg2: FW – TAGGGTAATCCCCTCCCTGC; RV - AGCAAGCCAGCTTCTCGAAT
Lyz: FW – GGATGGCTACCGTGGTGTCAAGC; RV - TCCCATAGTCGGTGCTTCGGTC
Reg3β: FW – TGGGAATGGAGTAACAAT; RV - GGCAACTTCACCTCACAT
Reg3ɣ: FW - CCATCTTCACGTAGCAGC; RV - CAAGATGTCCTGAGGGC
Gip: FW – AACTGTTGGCTAGGGGACAC; RV - TGATGAAAGTCCCCTCTGCG
Gcg: FW – CTTCCCAGAAGAAGTCGCCA; RV - GTGACTGGCACGAGATGTTG
Pyy: FW – ACGGTCGCAATGCTGCTAAT; RV - GCTGCGGGGACATCTCTTTTT
Sst: FW – GACCTGCGACTAGACTGACC; RV - CCAGTTCCTGTTTCCCGGTG
Sct: FW – GACCCCAAGACACTCAGACG; RV - TTTTCTGTGTCCTGCTCGCT
Cck: FW – GAAGAGCGGCGTATGTCTGT; RV – CCAGAAGGAGCTTTGCGGA
ChgA: FW – GTCTCCAGACACTCAGGGCT; RV - ATGACAAAAGGGGACACCAA
Saa1: FW – GGTCTTCTGCTCCCTGCTC; RV - AGCAGCATCATAGTTCCCCC
Saa2: FW – CAGCCTGGTCTTCTGCTCC; RV - CACATGTCTCCAGCCCCTTG
Saa3: FW – AGTAGGCTCGCCACATGTCT; RV - TCCATTGCCATCATTCTTTG

Transcriptome profiling

For transcriptome profiling, 400ng RNA was amplified and labelled using 3' IVT pico kit Affymetrix RNA amplification kit (Nugene) according to manufacturer's protocol. Microarray analysis of mouse EpCAM-positive cells was performed using Affymetrix Clariom® S 8-Array HT Plate according to the standard protocols of the Dutch Genomics Service and Support Provider (MAD, Science Park, University of Amsterdam, Netherlands). The data was normalised using Expression Console 1.4.1.46 and uploaded to R2: Genomics Analysis and Visualization Platform (http://hgserver1.amc.nl/). Microarray results were analysed using R2 software. Differentially expressed genes were selected based on fold change (≥ 2) in comparison to control group.

Software

nQuery 7.0: sample-size calculations
ImageJ: villi length and crypt depth measurement
Transcriptome Analysis Controle (TAC) and R2: microarray data analysis
LinRegPCR: quantitative Real-time PCR analysis
GeNorm: identification of most stable reference genes for quantitative Real-time PCR analysis
GraphPad Prism 8: statistical analysis and graph creation

Statistical analysis

Sample size was calculated using nQuery and based on maturation studies, using a two-group t-test of equal n's, with a significance level (α) of 0,05 and power of 80%. There were no exclusions or drop-outs. Data were analyzed using GraphPad Prism 8

and are presented as mean ± Standard Deviation unless stated otherwise in the figure legends. Sample distribution was determined using D'Agostino and Pearson normality test. Sample numbers, experimental replicates, type of statistical analysis test, and p values are reported in the figure legends.

Data availability

Microarray data are deposited in the Gene Expression Omnibus Database ().

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General Discussion and Future perspectives

PART I – MODELLING INTESTINAL EPITHELIAL MATURATION USING FETAL ORGANOIDS

Programmed to change

A healthy gut is the key to a healthy life. This has become the motto of current biomedical research. Almost every disease has been, in one way or another, connected to the health status of the gut, from cancer to autoimmune diseases and from metabolic to psychological disorders. Thus, understanding the functioning of the gut and how it develops has never been more urgent. In **chapter 2**, we contribute to this effort by developing a novel *in vitro* model of neonatal small intestine (SI) maturation, using fetal organoids which are derived from mouse primary fetal intestinal epithelial cells and maintained in a stable culture medium. These fetal organoids follow a similar maturation as the small intestinal tissue. This demonstrates that small intestinal epithelium maturation is an intrinsic process, confirming previous studies performed in the decades of the 80s and the 90s showing that mouse fetal intestinal isografts mature normally after transplantation into adult mice, i.e., without luminal signals or maternal hormones [1-3].

Our mouse fetal organoids are grown in EGF-Noggin-R-spondin (ENR) culture medium supplemented with growth factors, which is widely used for the growth of adult intestinal organoids. These factors could trigger the suckling-to-weaning reaction *in vitro*, but our observations indicate otherwise. First, the *in vitro* maturation occurs at a similar time frame as *in vivo* (**Chapter 2**). If the factors present in the culture medium would induce the typical developmental changes in gene expression and enzyme activity, they would occur some days or even hours after starting the culture, and not only two to three weeks later. Besides, enterocytes, enteroendocrine, and goblet cells were present throughout the one month of culturing fetal organoids, while Paneth cells appeared only after 14 days, mimicking the *in vivo* situation, and not earlier when fetal cells became exposed to the culture medium factors (**Chapter 2, Figure EV4F-H and Appendix Figs S2 and S3**). Therefore, the maturation we observe in our mouse fetal organoid model is likely not dependent of the culture medium but indeed an intrinsic programmed process.

Intrinsically regulated processes are present at many moments of the organism's development, starting at the very early embryo. There are several aspects of early embryonic development similar to intestinal development. First, a genome of an embryo is activated for the first time by an extremely precise and controlled sequence of events [4]. Some genes start to be activated first but the major embryo genome activation occurs with the start of the transcription of housekeeping genes. This genome activation occurs in a stepwise manner. For example, housekeeping genes start to be transcribed during the 2-cell and 4-cell stages at a low level, but at the 8-cell stage, their transcription is

sharply upregulated. Similar gradual gene activation over time also happens during the suckling-to-weaning transition (**Chapter 2 Fig EV2 and EV3**). Second, embryo genome activation seems to be regulated by changes in histone proteins and chromatin structure, accompanied by changes in transcription factor content or activity [4]. In turn, these changes are regulated by cell cycle-dependent mechanisms, in a coordinated fashion between the nucleus and cytoplasm. Indeed, the involvement of histone and chromatin remodulation in intestinal development has been recently described [5-7]. Third, during genome activation of the embryo, there is a shift in promoter utilization, which leads to different genes being transcribed [4]. Such a promoter shift might also play a role in the switch in expression in the SI from neonatal to adult markers. Lastly, embryonic cells sense changes in morphology and transduce these mechanical cues into biochemical signals that result in changes in gene and protein expression [8]. The emergence of villi in the intestinal epithelium at embryonic day (E) 16.5 is also connected with the start in the expression of intestine-specific genes in the epithelium [9]. It could well be that the appearance of crypts at postnatal day (P)14 also contributes to the changes in gene expression during the suckling-to-weaning transition.

A 'master switch' or a cascade of signals?

The major regulator of the suckling-to-weaning ever described is B lymphocyte-induced maturation protein 1 (Blimp-1) [10]. Blimp-1 is highly expressed in the neonatal intestine, delaying its maturation. Between P14 and P21, Blimp-1 expression is gradually lost, hand-in-hand with the gradual functional changes of the suckling-to-weaning transition. This step-by-step process is, once more, very similar to what is observed in early embryo development. Embryonic genome activation also does not occur as a 'master switch' but as a sequence of events initiated by DNA replication, that is followed by chromatin remodeling allowing the transcription and regulation of certain genes [4]. An identical process might explain how Blimp-1 controls both morphological transformations (crypt formation and Paneth cell appearance, disappearance of vacuolated enterocytes, microvilli development) and functional changes (decrease in expression of neonatal markers and increase in expression of adult markers) related to the suckling-to-weaning transition. Blimp-1 is a transcription repressor and is involved in histone arginine methylation [11]. Therefore, chromatin remodeling might also be the mechanism behind all the maturation hallmarks controlled by Blimp-1. The described fetal intestinal organoids can be used to investigate how Blimp-1 controls maturation. For example, adult organoids overexpressing Blimp-1 could not revert the maturation status (data not shown). It would be interesting to overexpress Blimp-1 in fetal organoids instead, to see whether they retain immature characteristics, due to prolonged delay in epithelial maturation. Deletion of Blimp-1 in fetal organoids should induce premature maturation, as described *in vivo* [10], while organoids isolated from KO Blimp-1 fetal mice will prob-

ably have adult characteristics. Such studies can easily be performed using our organoid model of intestinal maturation.

A sea of possibilities

This thesis highlights the great differences between neonatal and adult intestines, which can be fully reproduced by fetal and adult organoids. Intestinal studies performed in adult mice or organoids cannot be extrapolated to neonates. Our mouse fetal organoid model provides a novel platform to study intestinal epithelial maturation and the interactions of different external factors with the neonatal epithelium. We used the synthetic glucocorticoid dexamethasone as a proof-of-principle to show that *in vitro* intestinal epithelial maturation can be similarly modulated by an external factor as observed *in vivo* (**Chapter 2**). We further used this model to study the effects of antibiotics on neonatal IECs *in vitro* (**Chapter 4**). Many other perinatal factors can be studied using mouse fetal organoids, such as different types of milk or formula, breastmilk components, food nutrients, microbes, microbial products, pre/pro/symbiotics, hormones, drugs, among others. To more closely mimic the biological situation in such studies, it is important to isolate the organoids from the right developmental time point. We used late embryonic stage intestine, just prior to birth, to avoid any influence of breastmilk or microbe's exposure on the maturation status of the organoids. However, depending on the factor studied and the research questions, it could be more relevant to isolate intestinal organoids from neonatal mice. Although the maturation capacity of organoids isolated after birth still has to be demonstrated, we expect them to follow a comparable time frame if isolated before the beginning of the suckling-to-weaning transition, i.e., postnatal day (P) 14. After this time point, a great portion of organoids will probably be similar to adult organoids and neonatal features harder to investigate.

A standardized model that can be upgraded

The mouse fetal organoid model described in this thesis is rather simple. Organoids allow the study of intestinal epithelial cells separately from the other components of the small intestine. However, the interactions between epithelium and these other components are essential for many studies. Therefore, co-cultures with immune cells, fibroblasts, endothelial cells, neural cells, microbes, or microbial products, such as short-chain fatty acids (SCFA), can generate a more complex organoid system and allow the investigation of such interactions. Nevertheless, it is necessary to have a standardized protocol for these studies. We have published a precise, robust, and reproducible protocol for the growth of mouse fetal organoids, which culture conditions can be adjusted (**Chapter 3**). For example, to allow the growth of microbes, penicillin and streptomycin can be removed from the medium, and oxygen concentration can be reduced by using hypoxic chambers.

We have used several techniques to analyze fetal intestinal organoids (qRT-PCR, microarray, immunohistochemistry, immunofluorescence, enzyme activity, and seahorse). But other approaches can be used such as proliferation/apoptosis/viability studies by fluorescence-activated cell sorting (FACS), protein quantification by Western Blot, metabolic analysis of supernatant, and even methylation and acetylation studies, expanding the potential of this *in vitro* model.

Organoids have a 3D structure in which the apical side of the intestinal cells is turned towards the lumen of the organoid and the basolateral side is in contact with the Matrigel and the culture medium. Sometimes, it is important to access both apical and basolateral sides separately. We have successfully cultured organoids in a 2D monolayer using transwells (Figure 1A). Mouse fetal intestinal organoids can be dissociated into single cells using a trypsin-like reagent and then cultured on the membrane of a transwell coated with a thin layer of collagen or matrigel (protocol adapted from [12]). The transepithelial electric resistance (TEER) between the luminal and the basolateral side

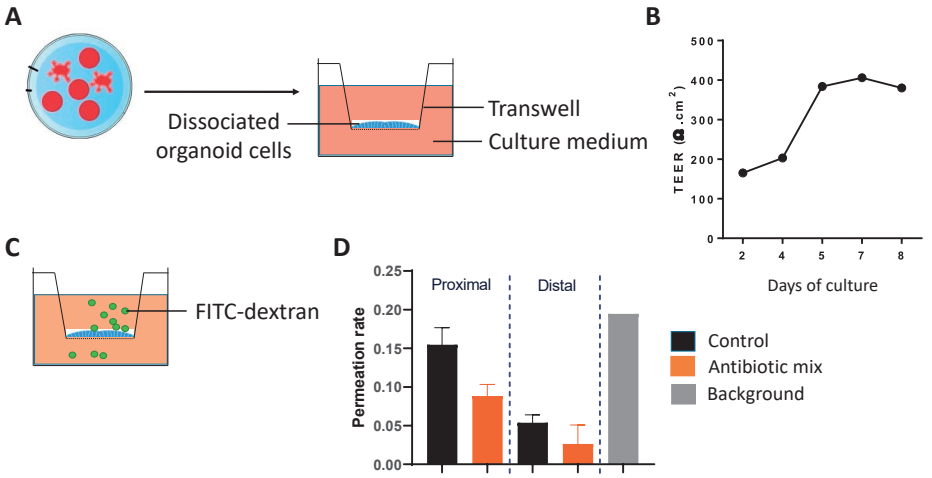

Figure 1 – Mouse fetal organoids can be cultured as a monolayer using transwells. A – Mouse fetal organoids can be dissociated into single cells, plated on a transwell covered by a layer of collagen or matrigel, and cultured in the same organoid culture medium used for organoids. **B -** TEER of the mouse intestinal fetal monolayer measured over time shows the initial increase of electric resistance and the plateau reached when the transwell is covered by cells and the monolayer is completely closed. The TEER is measured using an epithelial volt-ohm meter, which consists of two electrodes positioned on each side of the transwell/monolayer, without touching the cells. **C –** FITC-dextran can be used to assess the permeability of the monolayer. Cells are incubated with culture medium containing FITC-dextran on the apical side of the transwell and after 3-4 hours the amount of FITC-dextran that crossed the monolayer to the basolateral medium is measured. **D –** Example of the permeation rate (ratio basolateral/apical FITC-dextran concentration) measured in cell monolayers grown from proximal and distal mouse fetal intestine organoids. Antibiotics were added to the culture medium on day 7 of transwell culture and the assay was performed on day 11. Background indicates the value measured in a transwell without cells.

of the transwell increases and stabilizes with the closure of the monolayer (Figure 1B). The permeability of the *in vitro* fetal monolayer can also be assessed using fluorescein isothiocyanate-dextran (FITC-dextran) (Figure 1C and 1D FITC).

The hallmarks of intestinal maturation and suckling-to-weaning transition are identical between mice and humans, despite differences in duration and maturation status at birth. Nevertheless, using human fetal intestinal organoids would be a preferable choice. Preliminary data in our group has shown that organoids isolated from the human fetal small intestine do not have the same capacity to mature as the mouse fetal organoids. A reason for this could be the early developmental stage of the starting material. The human small intestinal tissue available for research originates from terminated pregnancies, which occur between 12 and 20 weeks of gestation. Contrary to the mouse intestine, at this stage, some of the adult intestinal characteristics are already detectable, as for example Paneth cells. However, they only reach their full defense capacity and number at birth. Based on previous studies showing that mouse intestinal organoids isolated at early developmental stages do not mature and also based on our work demonstrating that mouse organoids from late developmental stage intestine can mature, we hypothesize that <20 weeks of gestation human small intestine is still too early to isolate organoids capable to mature *in vitro*. Because obtaining human fetal intestine from late developmental stage is virtually impossible, especially tissue that has not yet been in contact with milk or microbes, our group is investigating which early factors can induce *in vitro* maturation of human fetal intestinal organoids.

Maturation and differentiation are different processes

The intestinal epithelium maturation occurs as two parallel processes. On one hand, the epithelium undergoes morphological changes, such as the development of villi and crypts. On the other hand, the epithelium experiences functional modifications, at the enzymatic and metabolic level, many concentrated at the brush-border. It is important to underline that these developmental changes are different from cell differentiation. Cell differentiation of IECs occurs when a stem cell leaves the crypt and migrates along the crypt-villus axis while acquiring the characteristics specific for a certain cell type, regulated by specific signaling pathways. Nevertheless, both intestinal epithelial maturation and differentiation go hand-in-hand, as most of the changes during the suckling-to-weaning transition occur also from crypt to villus (**Chapter 2, Figure EV2 and EV3**). In intestinal organoids, the formation of crypt-like budding structures reflects the presence of adult stem cells capable of differentiating into the different cell types [13]. However, an increase in the number of buds per organoid can represent either the formation of crypt-villus units characteristic of the mature epithelium or increased stem cell activity and differentiation. For example, we demonstrated that budding organoids are not a

synonym of maturation by showing protein expression of fetal and adult markers in both spheroids and organoids at early culture and at late culture time points, respectively (**Chapter 2, Figure 5C-E**). Thus, quantifying the number of buds should always be accompanied by other assays that more precisely analyze maturation markers to discern between intestinal maturation and differentiation.

The future of research: reducing animal use

The use of mouse intestinal fetal organoids considerably reduces the number of animals needed for research. Fetal organoids allow analysis over time of the intestinal maturation process, which is only possible *in vivo* if several animals are sacrificed at different time points. To further reduce the number of animals needed for organoid isolation, it is possible to use the whole intestine instead of separating the proximal and distal regions. When culturing proximal and distal organoids separately, the amount of intestinal material needed to start the cultures doubles. By using the whole intestine instead, i.e., culturing proximal and distal organoids together, less intestinal material is needed and, thus, fewer animals. In addition, fewer time points can be analyzed during the culture, so fewer wells are necessary and consequently fewer fetal intestines are needed for the culture.

Adult intestinal organoids treated with Wnt acquire a spheroid shape and have been described as fetal-like organoids because they upregulate the expression of embryonic genes [14]. Therefore, it may be hypothesized that this method could further reduce the number of animals needed to study fetal intestinal maturation, including the intestinalization process, as one adult small intestine yields significantly more organoids than one fetal small intestine. However, such organoids do not recapitulate the suckling-to-weaning transition and can not be used to study postnatal intestinal maturation.

PART II – SHORT- AND LONG-TERM EFFECTS OF EARLY LIFE ANTIBIOTICS ON THE SMALL INTESTINE

Is accelerated maturation a bad thing?

Events occurring in the first year after birth can affect our health later in life. This is especially relevant for our intestine. Several factors present in early life interfere with the normal maturation of the intestine and leave long-lasting marks. One of these factors are antibiotics (AB), which are the most frequently prescribed drug in infants and young children [15-17]. In this thesis, we show that AB treatment before weaning accelerates the maturation of the mouse small intestine (**Chapter 4, Figures 4 to 6)**. The key hallmarks of intestinal maturation, which occur earlier than usual after AB treatment, are

the disappearance of vacuolated enterocytes, reduction in permeability, downregulation of neonatal gene expression, upregulation of adult gene expression, and appearance of Paneth cells (PCs). These effects partially result from the direct action of AB on the intestinal epithelial cells (IECs), as demonstrated by the treatment of mouse fetal organoids with the same AB (**Chapter 4, Figures 8 and 9**).

Inadequate intestinal maturation in neonates (particularly in preterm newborns) can lead to necrotizing enterocolitis (NEC), a devastating intestinal disease resulting from inflammation, bacterial invasion, and consequent necrosis and perforation of the intestinal tissue, with a mortality rate of up to 50% [18]. Available literature concerning the association between AB use and NEC is inconsistent but the majority of studies show that prolonged AB use is associated with a higher risk of developing NEC [19-25]. Yet, there is also evidence that AB treatment may decrease the risk of NEC [26]. These different outcomes may be related to different types of AB used and/or different routes of AB administration (enteral or parenteral) [27]. Furthermore, considering that NEC is a multifactorial disease, it is difficult to determine the exact contribution of AB treatment to NEC development.

The development of NEC is directly correlated with prematurity [18]. Interestingly, in more premature babies, NEC develops later after birth: NEC does not appear right at birth but is delayed. In less premature babies, NEC develops shortly after birth. In fact, after correcting for gestational age, the incidence of NEC increases at 28wks, peaks at 32 wks and decreases at older ages [28]. Recently, this has been proposed to be connected to the great increase in the number and functionality of PCs that usually take place in the third trimester of gestation [29]. PCs begin to develop at 13.5 weeks of gestation in humans but only reach their full defense capacity and number at birth, thus, preterm newborns have immature PCs. In fact, neonates with NEC have a lower number of PCs compared to age-matched controls [29]. Moreover, ablation of PCs in mice results in an NEC-like phenotype [29]. Our work shows that AB in early life induces an increase in the number of PCs and that it is a direct effect of AB on the intestinal epithelium (**Chapter 4, Figure 6 and 9**). Therefore, this could be an explanation for the protective capacity of AB in the development of NEC observed in some studies.

Here, it is important to consider the variables in AB treatment: type of AB, route of administration, duration of treatment, gestational age, and postnatal age at the start of the treatment. Different types of antibiotics will affect the IECs differently not only because they target distinct bacteria strains and bacterial processes (with corresponding indirect effects on IEC's) but also because they enter the cell via different paths and at different regions of the gut. For example, amoxicillin is mostly absorbed in the duodenum

and jejunum through porins, because of its hydrophilic nature [30]. Vancomycin is also taken up via porins but is mainly absorbed in the colon and less in the small intestine. Metronidazole is lipophilic so it can diffuse through the cell membrane and is mainly absorbed in the duodenum and jejunum [31]. In this thesis, we show that the combination of these three AB accelerates intestinal maturation, but it remains unclear whether this effect is caused by one of the three AB or by a combination of two or only when the three are used together. Our *in vitro* fetal organoid model provides a relatively easy and fast method to investigate this. The route of administration is also an important determinant of the AB effects. For instance, enteral AB improves gut function and prevents NEC in preterm pigs but these differences are not observed when AB are administered via the parenteral route [27]. While in our *in vivo* experiments AB were given enterally, fetal intestinal organoids contacted with AB via the basolateral side. Thus, this could also explain the differences between both approaches. It would be interesting to use the above-mentioned 2D monolayer model to directly treat the luminal side of IECs with AB to compare with our results. This way, treating young pups with AB intravenously, which causes high discomfort for the animals, can be avoided. Regarding the treatment duration, we chose to treat mice pups with AB starting at P10 for 10 consecutive days. The next step will be to start AB treatment at different time points, corresponding to different ages in human infants, and with different durations. The importance of age is also clear from the different responses between proximal and distal SI observed in our work, as maturation occurs in a proximal-to-distal wave. In the end, the ultimate goal is to find the right single or combination of AB that can be prescribed at a specific time window and with a maximum duration without contributing to the development of NEC, which would eliminate one of the multiple factors involved in its onset.

Consequences of increased intestinal sensing

Early life AB induced differentiation of enteroendocrine cells (EECs) (**Chapter 4, Figure 4, 6, and 9**). Little is known about the emergence of EECs during the development of the intestine, but some evidence shows that this type of secretory cell is present in the early embryonic stages. For example, secretin is detected in the intestinal epithelium since E14.5 [32]. Interestingly, when comparing the gene expression of EECs markers between P10 and 8 weeks old we observed that their expression usually increases over time in the proximal SI but decreases in the distal SI (Figure 2).

The expansion in the number of EECs upon AB treatment might lead to an increased intestinal sensing capacity. This could result in hypersensitivity and/or dysregulation of gut hormones secretion, with consequences for the organism's health. Indeed, AB treatment in the first week of life has been shown to increase the risk of infantile colic, a common gastrointestinal disorder associated with, among others, hormone dysregula-

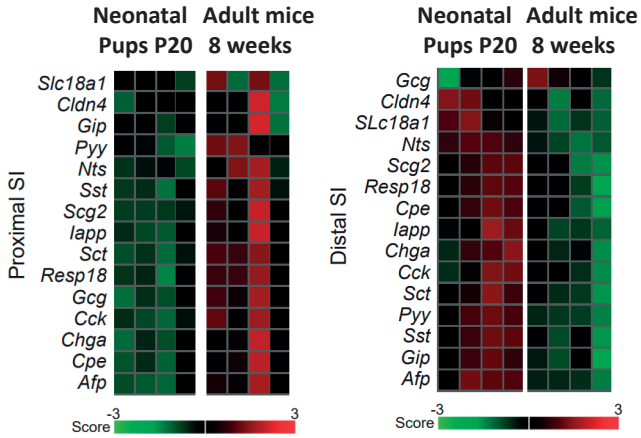

Figure 2 – Gene expression of enteroendocrine markers in proximal and distal SI epithelial cells from neonatal pups at P20 and 8 weeks old mice, measured by microarray.

tion and food hypersensitivity [33, 34]. Besides, the hormones secreted by EECs regulate several processes, such as food intake and digestion. The major consequences of unbalanced food intake and digestion are obesity and diabetes, which development has been associated with ABs in early life [35, 36]. We observe an increase in EEC upon AB but high EEC hormone concentration is linked with healthier body weight [37]. So how does AB treatment contribute to obesity and diabetes? First, it is essential to investigate whether the observed increase in enteroendocrine cell numbers goes hand-in-hand with an increase in hormone secretion. For example, the secretion of GIP, GLP-1, and PYY is completely abolished by Glut2 inhibition and, in our analysis at P20, Glut2 is downregulated in proximal epithelial cells of pups treated with AB (**Chapter 4, Supplementary Table 2**) [38]. Next, we need to understand the consequent effects of high hormonal concentration during such a vulnerable time: do they lead to alterations in the gut-brain axis and dysregulation of the normal pathways for food regulation? Finally, the increased risk of obesity linked to AB treatment in early life is a result of more factors than just changes in EEC, namely metabolism and microbiome [39]. Indeed, we also detected alterations in lipid metabolism in IECs in our study (**Chapter 4, Supplementary Table 1**). In addition, the microbiome and EEC can modulate each other. It is known that the metabolites produced by gut microbiota (including SCFAs, secondary bile acids, and lipopolysaccharides) can stimulate EEC [40], while CCK secreted by EEC prevents enteric bacterial overgrowth and translocation [41]. Interestingly, 28 different olfactory receptors (Olfr) were downregulated in the distal SI of 8-week-old mice that received early life AB. Little is known about the function of these receptors in the SI but they have been associated with enteroendocrine cell functioning and fat metabolism [42-45]. Therefore, olfactory receptors could also play a role in the development of obesity. Only by integrating all

these changes caused by AB and considering that they will be different in the neonatal intestine compared to the adult intestine, we will be able to comprehend the link between AB and the onset of obesity.

It all comes down to evolution

Evidence indicates that eukaryotic cells, like the ones composing our bodies, evolved from the symbiotic association between two prokaryotic cells, like bacteria, a hypothesis known as the endosymbiotic theory [46]. In the case of the animal cell, an anaerobic bacterium engulfed an aerobic bacterium, providing the ability for oxidative metabolism. In time, this gave rise to the mitochondria and the oxidative phosphorylation, or respiration, process. It is thus not surprising that AB can also target the mitochondria inside the host cells [46]. In this thesis, we show that AB treatment reduces the respiration capacity of mouse fetal organoids originating from the distal small intestine. Based on literature, we hypothesize that vancomycin inhibits the mitochondrial complex I leading to depolarization of the mitochondrial membrane. To prove this, the following set of experiments could be performed. First, seahorse analysis should be performed after treating fetal organoids with each AB separately or with different combinations of two AB, in order to understand whether the observed effects are caused by one single AB or a combination of two or three. Next, the activity of the mitochondrial complex I should be detected by specific assays. Alternatively, oxidative phosphorylation levels of vancomycin-treated fetal organoids should be compared to rotenone-treated fetal organoids, as rotenone is a potent specific inhibitor of the mitochondrial complex I. In addition, the integrity of the mitochondria should also be assessed using staining probes, such as MitoTracker. Because vancomycin treatment of epithelial cells has also been shown to increase the production of reactive oxygen species (ROS), hydrogen peroxide and nitric oxide concentrations should be quantified. Finally, it would also be interesting to investigate whether the effects are specific for the neonatal IECs by analyzing mouse adult organoids treated with the same AB.

The genetic and morphological changes we observed in the neonatal intestinal epithelium after AB treatment can only take place if alterations occur in the intestinal stem cells. Changes in metabolism can indeed signal stem cells to behave differently. Such a signal could be the trigger for earlier maturation of the IECs. It was previously shown that adult organoids have lower glycolytic capacity compared with Wnt-induced fetal organoids [14]. Therefore, the reduced glycolytic capacity and glucose intake we observed in proximal and distal fetal organoids treated with AB might trigger their maturation. To demonstrate this, glycolysis could be pharmacologically inhibited in fetal organoids to analyze whether adult maturation markers, Paneth cells, and enteroendocrine cells also increase as observed in AB-treated fetal organoids.

Discovering the exact mechanism connecting changes in metabolism with epithelial maturation would be the next step. We could start by examining the mechanistic target of rapamycin (mTOR) signaling, which plays a central role in intestinal epithelial regulation when metabolic changes occur. The involvement of the Wnt pathway could also be investigated, as Wnt regulates intestinal stem cell proliferation and Paneth cell differentiation [47]. Besides, the leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5), the specific marker for intestinal stem cells and enhancer of Wnt signaling, increases in expression as PCs emerge and becomes confined to the crypt region during the suckling-to-weaning transition (**Chapter 2 Appendix Figure S2**). Thus, Wnt signaling might also help integrate the metabolic changes into epithelial modifications which result in accelerated maturation. Another mechanism that should be considered is epigenetics. Alterations in the mitochondria, including mitochondrial complex I dysfunction, can result in modifications in DNA acetylation and DNA methylation, for example by influencing TET enzymes activity [48, 49]. Ultimately, this can affect gene expression and cell differentiation [50].

Our metabolic observations in fetal organoids also need to be confirmed *in vivo*. Although it is remarkable that AB can directly affect IECs, the situation might be different when considering the whole intestinal tissue and interactions with the microbiome.

The elephant in the room

There are trillions of bacteria from more than 2000 different species inside our bodies. In fact, 90% of the cells in our bodies are bacterial. Contrary to the common belief, bacteria are not only important in the colon but also in the small intestine, from the duodenum to the ileum, in both children and adults [51-55]. For instance, bacteria present in the duodenum regulate digestion and absorption of lipids, as shown in germ-free mice (GF): GF mice fed a high-fat diet were not able to absorb fat and maintained their original weight, while specific pathogen-free (SPF) mice were able to absorb fat and gained weight [56].

In recent years, it has become clear that a healthy gut can be attributed to a more diverse and resilient commensal microbiome. The initial colonization of the gut is controlled by multiple factors such as type of birth (vaginal versus cesarian), diet (breast milk versus formula with/without prebiotics, milk oligosaccharides, etc.), and intestinal epithelial defense status. This results in a highly variable gut microbiota composition between individuals, which is further influenced by their nutrition, age, and geography. This helps explain why the effects of ABs on the microbiota are highly individualized and can even vary between repeated exposure to the same AB. Besides, ABs do not only affect the bacterial strains they target but also the bacteria that are co-dependent from the

affected strains [57]. Therefore, it is remarkably difficult to pinpoint which strains are affected by each AB. It is important to note that effects on SI microbiota are different from fecal microbiota effects, so comparisons between studies should be cautious [58].

Although the effects of AB treatment on intestinal microbiota vary substantially between individuals, its diversity and amount are always reduced. ABs also alter microbiota's metabolism and their capacity to resist pathogens. Together, these effects contribute to the onset of all kinds of diseases. For example, obesity has been linked to a decrease in Bacteriodetes and an increase in Firmicutes, which can be triggered by AB, and lead to higher extraction of SCFAs from fiber and consequent increase in energy harvesting [59]. ABs can also alter the oxygen gradient in the SI, increasing the oxygen concentration in the lumen and facilitating the colonization and proliferation of aerobic bacterial strains/pathogens, which will then influence the metabolism and function of the epithelial cells [60]. In our work, many of the differences observed in the SI epithelium of AB-treated pups compared to control pups were not replicated in the AB-treated mouse fetal intestinal organoids, indicating that they result from the effect of AB on the microbiota, which in turn affects the IECs. One example is the upregulation of Reg3g expression in IECs upon AB treatment (P20) but not in the fetal organoids *in vitro*. This is in agreement with recent findings demonstrating that regulation of Reg3g in IECs is associated with the balance between commensal and opportunistic gut bacteria [61].

An impact for life?

Determining the mechanism behind the changes observed in the SI epithelium upon AB treatment, i.e., P20 will help understand whether the effects of AB in early life are sustained throughout adulthood. One could speculate that if these effects result, at least partially, from alterations to the epigenome of SCs/IECs, these could be long-lasting. Early life SI development is regulated by DNA methylation and histone acetylation and ABs have been shown to affect intestinal epithelial DNA methylation in preterm pigs [7, 62]. Thus, it is plausible that ABs affect the epigenome of the SI epithelium leading to its accelerated maturation and increased differentiation of Paneth and enteroendocrine cells.

Besides morphological differences, especially in the distal SI, the main contrast between adult mice that received AB in early life and control mice was the differential expression of defense markers. However, we did not observe any health issues in the mice during the 5 weeks after AB treatment. Both control and AB-treated mice were healthy and kept in controlled conditions throughout the experiment. The question is whether these adult mice, which have reduced intestinal defense caused by AB treatment in early life, will respond differently to later life challenges. It would be interesting to repeat these

experiments while challenging the gut in adulthood, by for example, restrain stress or dextran sulfate sodium (DSS), to determine the negative long-term effects of AB in early life.

PART III – REDUCING ANTIBIOTIC USE AND ITS EFFECTS

The holistic approach

Antibiotics are life-saving drugs for many neonates, especially in case of premature birth which is associated with a higher risk of sepsis. Nevertheless, many neonates receive AB without culture-proven infections. Our results reinforce the urgency in reducing the amount of AB prescriptions and the duration of AB treatment. Recently, it has been shown that using the neonatal early-onset sepsis (EOS) calculator reduced empiric antibiotic treatment of term neonates by more than 40% compared to conventional assessments [63]. Based on the EOS calculator, newborns are stratified into three different risk levels depending on five maternal and four neonatal clinical risk factors [64]. Furthermore, reducing the duration of intravenous antibiotic treatment from 10 to 7 days results in shorter hospital stay and does not seem to increase treatment failure [65]. Such efforts should be confirmed and translated to official international guidelines.

The health of an individual is not only dependent of its genome. External factors can directly or indirectly affect human health. The exposome includes all factors present in the environment surrounding an organism, but also the environment of the planet, which contribute to the degradation of its health. These include lifestyle (diet, physical activity, sleep, smoking), social (inequality, income, stress), ecosystem (food accessibility, population density, green space), and physical-chemical factors (temperature, pollution) [66]. A healthier lifestyle may also contribute to a lower risk of preterm birth and therefore to reduced use of ABs [67, 68]. Therefore, a healthier exposome can ultimately lead to fewer ABs being prescribed to infants and children.

Fecal microbiota transplantation

After discovering the influence of the gut microbiome on disease development, researchers turned their hopes to fecal microbiota transplantation (FMT), which essentially provides a patient with a sample of microbiota from a healthy donor. The cure rate of FMT on recurrent *Clostridioides difficile* infections is 90% [69]. However, it has been challenging to achieve such a high success rate for other diseases, such as inflammatory bowel diseases (IBD) and irritable bowel disease (IBS) [69]. Evidence shows that not only the donor sample is crucial for the efficacy of the FMT, but also the status of the recipient is determinant for its success [69]. Nevertheless, preclinical mouse studies

suggest that in the future this could be an option to consider for children that need to receive AB treatment. In fact, experiments in animal models seem to indicate that their mothers could be the perfect donors. It has been recently shown that the development of diabetes type 1 associated with early life AB treatment can be partially avoided by maternal cecal microbiota transfer (CMT) to NOD mice pups [61]. Maternal CMT also rescues the expression of some innate defense genes affected by AB treatment in the ileum of NOD mice at P42, such as Reg3g. Of note, the expression of Reg3g was downregulated in the ileum of NOD mice at P23 after AB treatment (between P5 and P10), while in this thesis we report upregulated Reg3g expression upon AB, highlighting the variation in outcomes depending of the type and duration of antibiotics, starting time, mouse strain, and animal facilities. In addition, CMT also reverted AB-induced alterations in histone post-translational modification and microRNA expression in the ileum. However, the expression of some genes like Saa1/2 could not be rescued by CMT. In our experiments, Saa1/2 was also significantly downregulated in the ileum of adult mice treated with early life AB (**Chapter 5 Figure 5**). These genes are inversely correlated with the development of colitis [70, 71], so CMT might not completely protect the ileum from colitis. Nevertheless, it could be a promising approach to revert the negative effects of AB treatment, although this hypothesis would still need extensive research.

Pre-, pro- and symbiotics: the antidote to antibiotics

The use of non-food prebiotics, probiotics, and synbiotics has increased in both adults and children from 1999 to 2018, in the U.S. [72]. The prebiotic concept was formally introduced in 1995 and is currently defined as “a substrate that is selectively utilized by host microorganisms conferring a health benefit” [73]. The most widely studied prebiotics are the simple fructans and galactans, available commercially as inulins, fructooligosaccharides (FOS), and galactooligosaccharides (GOS) [74, 75]. Prebiotics provide health benefits through several different mechanisms, including stimulation of growth, and/or activity of health-promoting bacteria similar to human milk fed infants, reduction of harmful bacteria presence, and lower incidence of infections and allergyic symptoms [76-79]. Bifidobacteria and lactic acid-producing bacteria can ferment prebiotics and produce SCFAs, such as butyrate, acetate, and propionate, which reduce the local pH, induce the production of immunomodulatory cytokines, stimulate mucin production, and function as an energy source for the host IECs [74, 80, 81]. Importantly, prebiotics can directly regulate the integrity of the intestinal cell barrier and could, thus, compensate for the direct effects of AB on IECs described in this thesis [75, 80]. When given during pregnancy and lactation in mice, prebiotics improve offspring’s body weight, muscle and bone mass gain, increase colon length, and reduce allergy and asthma symptoms [82]. Supplementing neonatal rats with fructo-oligosaccharides or short-chain galacto-oligosaccharides/long-chain fructan mix (scGOS/lcFOS) between P5 and P14 leads to

a reduction in Firmicutes and increase in Bifidobacteria, bacteria linked to a healthier gut [83]. Human milk oligosaccharides (HMOs) are often considered naturally occurring prebiotics. Although the infant's gut is not able to digest HMOs, they are abundant and diverse in human milk. HMOs stimulate certain bacterial strains' growth, including Bifidobacteria and Bacteroides [84]. Infants fed formula supplemented with HMOs (2'-fucosyllactose and lacto-N-neotetraose) have less chance of requiring antibiotics during the first week of life compared to infants receiving only formula [85]. Therefore, scGOS incombination with lcFOS and HMOs are currently added to infant formulas [86]. On the other hand, prebiotics do not seem to prevent NEC development [80, 84].

Probiotics are defined as "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" [87]. Common probiotics used in neonates and infants are bifidobacteria and lactobacillus [80]. In contrast with prebiotics, probiotics seem to help to prevent NEC and reduce systemic and intestinal inflammation in neonates [74, 88, 89]. It has been hypothesized that these benefits result from the capacity of probiotics to regulate innate and adaptative immunity and modulate intestinal tight junctions and signaling pathways in IECs, which affect the integrity of the intestinal barrier [80, 90]. In addition, *in vitro* exposure to probiotic condition media (*Bifidobacterium infantis* and *Lactobacillus acidophilus*) attenuated inflammatory response in immature human enterocytes (fetal SI 12-20 weeks of gestation ileum), in immature human intestinal xenografts (fetal ileum), and primary enterocyte culture of NEC tissue (neonatal 25 weeks of gestation ileum) [91]. Still, the benefits of probiotics are dose-dependent and species-specific [80]. Thus, further investigation is necessary to confirm the potential of probiotics on the mitigation of AB effects in early life.

A third approach is to combine prebiotics and probiotics, or also termed synbiotics. Synbiotics are "a mixture comprising live microorganisms and substrate(s) selectively utilized by host microorganisms that confers a health benefit on the host" [92]. Synbiotics either have a complementary effect, when their components are independently chosen so each has its specific effect, or a synergistic effect, when the prebiotic supports the growth of the probiotic increasing the latter's efficacy [74]. Synbiotics seem more helpful in preventing NEC than probiotics alone [80]. Mice that received synbiotics (scGOS/lcFOS with *Bifidobacterium breve M-16V*) in early life until P42 and were then challenged with a high-fat Western-style diet for 8-weeks, showed less excessive fat accumulation, improved insulin sensitivity and dyslipidemia, and altered gene expression in the ileum, mostly related to lipid and cholesterol metabolism, compared to control mice that did not receive synbiotics [93]. However, transplantation of microbiota from synbiotic-supplemented to control mice at P42 did not reproduce the same beneficial results. This suggests that the long-lasting protection given by synbiotics does not de-

pend of alterations in the microbiota but might result from the direct regulation of the intestinal cells by synbiotics [93].

FINAL REFLECTION

In summary, the novel *in vitro* model of intestinal postnatal maturation described in this thesis can be used in the future to investigate the mechanisms behind the regulation of the suckling-to-weaning transition and the interaction of perinatal factors with neonatal intestinal epithelial cells. We propose that the use of mouse fetal organoids in co-cultures with other intestinal components or cultured as a 2D monolayer, and eventually the use of human fetal organoids that can also mature *in vitro*, will largely reduce animal experiments and provide observations with higher translational value.

This thesis provides some more pieces to the puzzle, by identifying the direct and indirect effects of AB on the small intestine of mice and intestinal organoids. Still, our findings left many unanswered questions. In our opinion, the next steps should determine which antibiotic(s) is(are) causing the effects described in our work and whether different routes of antibiotic administration or treatment duration can minimize these effects. Furthermore, it is essential to uncover the mechanisms of direct antibiotic action on the intestinal epithelial cells, with a special focus on metabolism and epigenetics. The possible link between accelerated intestinal epithelial maturation induced by antibiotics and NEC development, as well as the potential association between increased presence of enteroendocrine cells and the onset of infantile colics and obesity/diabetes should also be elucidated. In addition, how changes directly induced by antibiotics on the neonatal epithelium correlate with differences in adulthood under challenging conditions remains to be investigated. Finally, the capacity of HMOs, pre-, pro-, and synbiotics of reverting the effects of early life antibiotics should be promptly studied.

Antibiotics will always be prescribed to (newborn) infants and children suspected of infection. Therefore, it is important to understand their full impact on the organism's function and development and how we can reverse or limit their detrimental effects We hope this work paves the way to further study the causal effect of AB in early life on the development of diseases in adulthood and how we can limit or even prevent these detrimental short- and long-term effects and improve the global health of future generations.

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Thesis Summary and Nederlandse Samenvatting

THESIS SUMMARY

The small intestine of an infant differs from the adult small intestine. During the first months of life, the intestinal cells are immature as they are fully prepared to digest milk, and not or less capable of digesting other types of nutrition. Moreover, because intestinal colonization with bacteria occurs after birth, the intestine is vulnerable to infections during this period. In the course of several months and up to two years, the small intestine continues its morphological and functional maturation and, after weaning, the intestinal cells finally acquire all the adult characteristics. This means that the intestinal cells are capable of digesting solid food and can defend themselves against pathogens. In some cases, this developmental process of intestinal maturation is affected, which can leave long-lasting consequences to health. One of the factors known to affect the postnatal development of the small intestine is antibiotics. Antibiotics are the most common drug prescribed to infants and children, due to the high incidence of bacterial infections in these age groups. However, the use of antibiotics has been associated with the development of diseases, such as inflammatory bowel diseases, obesity, and allergy, although the underlying mechanisms are still to be found. The lack of research models available to study postnatal intestinal development has limited advances in understanding how early life antibiotics impact later life intestinal health.

This thesis aimed to investigate the short- and long-term effects of antibiotic treatment during early life on the small intestine, with a special focus on the intestinal epithelium. **Chapter 1** shortly introduces the small intestine development and the current literature on the effect of early life antibiotics. In **chapter 2**, we established a model using mouse intestinal fetal organoids that allowed us to study intestinal epithelial maturation *in vitro*. Intestinal organoids are mini-guts constituted by all principal intestinal epithelial cell types, which can grow as three-dimensional structures supported by a gel scaffold. We found that mouse intestinal fetal organoids can mature in a culture dish following the same hallmarks and timeline as the small intestine in a living mouse, confirming that the postnatal intestinal maturation is specifically programmed in the intestinal epithelial cells. In a proof-of-principle experiment, we used dexamethasone to accelerate the maturation of the mouse fetal intestine organoids, similar to what has been shown in living mice. This demonstrated that the organoid model we developed can be used to study the effect of external factors on intestinal maturation. The standardized protocol, accompanied by a tutorial video and methods for intestinal maturation analysis, has been made available to the scientific community and is described in **chapter 3**.

We then proceeded with the identification of the changes induced by antibiotic treatment on the small intestine of neonatal mice. In **chapter 4**, we found that early life

antibiotics induced premature intestinal maturation. This was demonstrated by the presence of several maturation hallmarks in the small intestine of antibiotic-treated pups: decreased intestinal permeability, disappearance of neonatal vacuolated cells, emergence of Paneth cells, lower expression of neonatal markers, and higher expression of adult markers. Besides, early life antibiotics led to increased expression of enteroendocrine cell markers, especially in the proximal small intestine, and to the upregulation of epithelial defense markers, specifically in the distal small intestine. When treating mouse fetal intestine organoids with the same antibiotics we observed a similar increase in expression of adult markers and a higher number of Paneth and enteroendocrine cells. Therefore, we concluded that these effects resulted from the direct action of antibiotics on the immature intestinal epithelial cells, independently of the antibiotics-induced effects on the gut microbiome or other intestinal cell types. Finally, early life antibiotics also directly impaired the metabolic capacity of the fetal intestinal organoids.

To understand whether the identified antibiotic-induced effects in the neonatal intestine were still present later in life, we examined in **chapter 5** the small intestine of adult mice that received the same antibiotic treatment in early life. In the proximal small intestine of these mice, we found hyperproliferative crypts, increased number of Paneth cells, and altered expression of two enteroendocrine cell markers. In contrast to our observations in the antibiotic-treated pups, the expression of antibacterial defense markers in the adult distal small intestine was reduced. Taken together, these results suggest that antibiotic use in early life has long-term effects on gut functioning and may even contribute to the onset of diseases later in life.

In conclusion, this thesis demonstrates that mouse intestinal fetal organoids can be used as an *in vitro* model to study the influence of external factors on intestinal epithelial maturation. Further, our work revealed that antibiotic treatment in early life accelerates the maturation of the mouse intestinal epithelium, induces the differentiation of enteroendocrine cells, and reduces the metabolic capacity of mouse fetal intestinal organoids. Additionally, we showed that early life antibiotics impact the intestinal epithelium structure and functioning later in life. The next steps should determine the mechanism behind these effects and explain how antibiotic treatment in early life leads to the the development of certain diseases. Finally, future work should study whether human milk oligossacharides, pre-, pro-, or synbiotics are able to limit or even prevent the detrimental short- and long-term effects of antibiotics.

NEDERLANDSE SAMENVATTING

De dunne darm van een kind verschilt van die van een volwassene. Gedurende de eerste maanden na de geboorte zijn de darmcellen onrijp omdat zij voornamelijk gericht zijn op het verteren van melk en niet of minder in staat zijn tot vertering van andere voeding. Omdat de kolonisatie van de darm met bacteriën start na de geboorte is de darm gedurende deze periode vatbaarder voor infecties. In de loop van de eerste levensmaanden tot aan het tweede levensjaar ondergaat de dunne darm morfologische en functionele rijping en na het starten van vaste voeding ontwikkelen de darmcellen hun volwassen kenmerken. Daarmee zijn de darmcellen in staat om vast voedsel te verteren en zichzelf te verdedigen tegen pathogenen. In sommige gevallen wordt het proces van de darmrijping verstoord wat langetermijn gevolgen kan hebben voor de gezondheid. Een van de factoren waarvan bekend is dat het de postnatale ontwikkeling van de dunne darm verstoort zijn antibiotica. Antibiotica zijn de meest frequent voorgeschreven medicijnen bij zuigelingen en kinderen, door de hoge incidentie van bacteriële infecties in deze leeftijdsgroep. Echter het gebruik van antibiotica is ook gerelateerd aan de ontwikkeling van ziektes zoals inflammatoire darmziekten, obesitas en allergieën, hoewel de onderliggende mechanismen nog niet goed bekend zijn. Het gebrek aan beschikbare onderzoeksmodellen om de postnatale darmontwikkeling te bestuderen heeft de vooruitgang beperkt in het begrip over de manier waarop antibiotica, gegeven kort na de geboorte, de gezondheid van de darm in het latere leven beïnvloedt.

Het doel van dit proefschrift is om de korte- en lange termijneffecten van antibiotica te onderzoeken op de dunne darm gedurende het vroege leven, in het bijzonder op het darmepitheel. Hoofdstuk 1 geeft een korte introductie over de ontwikkeling van de dunne darm en de huidige literatuur over de effecten van antibiotica bij pasgeborenen. In hoofdstuk 2 wordt de ontwikkeling van een organoid model beschreven waarbij gebruik gemaakt wordt van foetale muizendarmen, die ons in staat stelt om de rijping van het darmepitheel *in vitro* te bestuderen. Darmorganoïden zijn mini-darmen opgebouwd uit alle belangrijkste epitheliale celtypes, die kunnen groeien als drie-dimensionale structuren in een kweekmedium. We vonden dat de foetale darmorganoïden uit muizen zich kunnen ontwikkelen in een kweekschaal met dezelfde kenmerken en tijdslijn als de dunne darm van een levende muis. Dit bevestigt dat de postnatale darm rijping specifiek geprogrammeerd is in de intestinale epitheliale cellen. In een proof-of-principle experiment hebben we dexamethasone gebruikt om de rijping van de foetale darmorganoïden uit muizen te versnellen, vergelijkbaar met die bij levende muizen. Dit demonstreert dat het door ons ontwikkelde organoid model gebruikt kan worden om de effecten van externe factoren op darmrijping te bestuderen. Het gestandaardiseerde protocol gecombineerd met een instructievideo en methoden voor de analyse van darm rijping

is beschikbaar gesteld aan de wetenschappelijke gemeenschap en wordt beschreven in **hoofstuk 3.**

Vervolgens zijn wij verdergegaan met het identificeren van veranderingen die worden veroorzaakt door antibiotica in de dunne darm van neonatale muizen. In **hoofdstuk 4** hebben wij vastgesteld dat gebruik van antibiotica bij pasgeborenen zorgt voor vroegtijdige darm rijping. Dit is aangetoond door de aanwezigheid van een aantal rijpingskenmerken in de dunne darm bij de antibiotica-behandelde muizenpups: afname van intestinale permeabiliteit, het verdwijnen van neonatale gevacuoleerde cellen, het verschijnen van Paneth cellen, hogere expressie van neonatale markers, en lagere expressie van volwassen markers. Daarnaast leidde antibiotica bij pasgeborenen tot een toename in de expressie van entero-endocriene celmarkers, met name in de proximale dunne darm, en tot een verhoging van epitheliale verdedigingsmarkers, met name in de distale dunne darm. Bij de behandeling van foetale darmorganoïden van muizen met dezelfde antibiotica werd een vergelijkbare toename geconstateerd in de expressie van volwassen markers en een hoger aantal Paneth- en entero-endocriene cellen. Op basis van deze bevindingen kunnen we concluderen dat deze effecten voortkomen uit de directe werking van antibiotica op de onrijpe epitheliale darmcellen, onafhankelijk van de door antibiotica veroorzaakte effecten op het darm microbioom of andere celtypes in de darm. Tenslotte leidden antibiotica bij pasgeborenen tot directe verslechtering van de metabole capaciteit van de foetale darmorganoïden.

Om te begrijpen of de door antibiotica veroorzaakte effecten in de neonatale darm ook nog aanwezig zijn in het latere leven, hebben we in **hoofdstuk 5** de dunne darm van volwassen muizen onderzocht die dezelfde antibioticakuur hebben ontvangen tijdens het vroege leven. In de proximale dunne darm van deze volwassen muizen vonden wij hyperprofileratieve crypten, toegenomen aantallen Paneth cellen en aangepaste expressie van twee entero-endocriene celmarkers. In tegenstelling tot onze observaties bij de met antibiotica behandelde muizenpups, was de expressie van antibacteriële verdedigingsmarkers in de volgroeide distale dunne darm verminderd. Samengevat wijzen deze resultaten erop dat het gebruik van antibiotica bij pasgeborenen langetermijneffecten heeft op het functioneren van de darmen en zou kunnen bijdragen aan het ontstaan van ziektes in het latere leven.

Concluderend toont dit proefschrift aan dat foetale muis-darmorganoïden kunnen worden gebruikt als een *in vitro* model om de invloed van externe factoren te bestuderen op rijping van het darmepitheel. Verder heeft ons werk aangetoond dat het gebruik van antibiotica bij pasgeborenen zorgt voor versnelde rijping van het darmepitheel, differentiatie van entero-endocrine cellen, en dat de metabole capaciteit vermindert van

foetale darmorganoïden uit de muis. Daarnaast hebben we aangetoond dat antibiotica bij pasgeborenen invloed heeft op de darmepitheel structuur én functioneren in het latere leven. Vervolgstappen zouden de mechanismen achter deze effecten moeten vaststellen en verklaren hoe antibioticagebruik bij pasgeborenen leidt tot de ontwikkeling van bepaalde ziektes. Tenslotte zou toekomstig onderzoek zich moeten richten op de vraag of humane melkoligossacharides, pre-, pro-, of synbiotica in staat zijn om de effecten van antibiotica (op korte en lange termijn) te beperken of zelfs helemaal te voorkomen.

CONTRIBUTING AUTHORS

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Department of Gastroenterology and Hepatology, Tytgat Institute for Intestinal and Liver Research, Amsterdam UMC, AGEM, University of Amsterdam, Amsterdam, The Netherlands
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PORTFOLIO

Name PhD student: Tânia Martins Garcia
PhD period: January 2016 – October 2020
Name PhD supervisor: Prof. Dr. Ruurd M. van Elburg
Names PhD co-supervisors: Dr. Ingrid Renes and Dr. Vanesa Muncan

1. PhD training

	Year	ECTS
Courses		
- Laboratory Animal Science	2016	3.9
- Oral Presentation in English	2017	0.2
- Biosafety and Biosecurity in general	2017 - 2020	0.1
- Biosafety Level 1 and 2 Environment	2017 – 2020	0.1
- Advanced qPCR	2018	0.9
Seminars, workshops and master classes		
- OASIS seminars	2016-2019	0.2
- Tager lectures	2016-2019	0.2
- Seminars in Gastroenterology and Hepatology	2016-2019	0.2
- 100 Years Hubrecht Institute Symposium	2016	0.3
- Workshop “Transferable Skills” by HFP Consulting	2017	0.3
- Amsterdam UMC Organoid Symposium	2017	0.1
- R2 Introduction Workshop	2018	0.1
- AGEM Symposium – *Nutrition* (Amsterdam)	2018	0.1
- Amsterdam Kindersymposium (online)	2021	0.1
Presentations		
- “Guts growing up in a dish”, ENABLE Symposium	2017	0.3
- “Mouse fetal intestinal organoids as a model for intestinal epithelial maturation”, Keystone Symposium	2017	0.5
- “Intestinal fetal organoids as a model of intrinsic postnatal epithelial maturation”, European Society for Paediatric Gastroenterology, Hepatology and Nutrition	2018	1.0
Congress & NVGE Digestive Disease Days		
- “Mouse fetal organoids: a new model to study epithelial maturation from suckling to weaning”, ISSCR Symposium	2019	0.5
- “Guts growing up in a dish”, FameLab competition	2019	1.0
- “Early life antibiotics influence *in vivo* and *in vitro* mouse intestinal epithelium maturation and functioning”, NVGE Digestive Disease Days	2020	0.8
(Inter)national conferences		
- Herrenhausen Conference - *The Neonatal Window of Opportunity, Early Priming for Life* (Hannover)	2016	0.8
- Nederlandse Vereniging voor Gastroenterologie (NVGE) Digestive Disease Days (Veldhoven)	2016/18/2020	1.5
- Keystone Symposium - *Gastrointestinal Control of Metabolism* (Copenhagen)	2017	1.5
- The European Academy for Biomedical Science (ENABLE) Symposium – *Breaking down Complexity: Innovative Models and Techniques in Biomedicine* (Barcelona)	2017	1.0
- Amsterdam Gastroenterology Endocrinology and Metabolism (AGEM) Institute Annual PhD retreat (Garderen)	2017 – 2020	2.0
- International Society for Stem Cell Research (ISSCR) Symposium – *Stem Cells & Organoids in Development & Disease* (Amsterdam)	2019	1.0

Other

- Peer to Peer Group Coaching	2019	0.5
- Journal Club	2016-2020	10
- Teaching sessions Tytgat Institute	2016-2020	1.2
- Progress Report Tytgat Institute	2016-2020	2.5

2. Teaching

	Year	**ECTS**
Mentoring		
- Teaching of laboratory techniques and revision of Master Thesis of Joost Wijnakker (NKI)	2019	0.5
Supervising		
- Emma Cats, *"The non-antibacterial effects of antibiotics and type of feeding in (pre) term infants: a systematic review"*	2017-2018	1
- Chahida Nafid and Jade Jansen, *"Neonates and Antibiotics"*	2017	0.1

3. Parameters of Esteem

	Year
Grants	
- Travel Grant ENABLE Symposium	2017
Awards and Prizes	
- Abstract prize, NVGE Digestive Disease Days	2020
- Jury FameLab competition, AMC	2020

4. Publications

	Year
In this thesis	
- Garcia TM, Navis M, Renes IB, Vermeulen JL, Meisner S, Wildenberg ME, van den Brink GR, van Elburg RM, Muncan V *Mouse fetal intestinal organoids: new model to study epithelial maturation from suckling to weaning.* EMBO reports. 20: e46221.	2019
- Garcia TM, Navis M, Wildenberg ME, van Elburg RM, Muncan V *Recapitulating Suckling-to-Weaning Transition In Vitro using Fetal Intestinal Organoids.* Journal of Visualized Experiments. 153: e60470.	2019
- Garcia TM, van Roest M, Vermeulen JL, Meisner S, Smit WL, Silva J, Koelink PJ, Koster J, Faller WJ, Wildenberg ME, van Elburg RM, Muncan V, Renes IB *Early Life Antibiotics Influence In Vivo and In Vitro Mouse Intestinal Epithelium Maturation and Functioning.* Cellular and Molecular Gastroenterology and Hepatology. 12(3): 943-981.	2021
- Garcia TM, van Roest M, Vermeulen JL, Meisner S, Koster J, Wildenberg ME, van Elburg RM, Muncan V, Renes IB *Altered Gut Structure and Anti-Bacterial Defense in Adult Mice Treated with Antibiotics during Early Life.* Antibiotics. 11(2): 267.	2022
Other peer reviewed publications	
- Smit WL, Spaan CN, de Boer RJ, Ramesh P, Garcia TM, Meijer BJ, Vermeulen JL, Lezzerini M, MacInnes AW, Koster J, Medema JP, van den Brink GR, Muncan V, Heijmans J *Driver mutations of the adenoma-carcinoma sequence govern the intestinal epithelial global translational capacity.* Proceedings of the National Academy of Sciences of the United States of America. 117(41): 25560-25570.	2020

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10 January 1992 in Lisboa
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PROFILE

Versatile, hard-working, precise and reliable

Demanding and fast-learner

Always looking for new challenges

Passionate, honest, good-listener and open-minded

Full of ideas and initiatives

SKILLS

Communicative and confident public speaker

Dedicated guide and trainer

Event planner and organizer

Portuguese, English, Spanish (fully proficient), Dutch (B2)

ACHIEVEMENTS & EXPERIENCE

2021

Project Manager at The TIM Company

Assoc. Editor Human Nutrition & Metabolism

5 yrs

PhD with Vanesa Muncan, Ingrid Renes and Ruurd van Elburg
Tytgat Institute, Amsterdam UMC (ISO 15189) & Danone Nutricia Research
Effects of early antibiotics on gut maturation and functioning

Course Introd. to Proj. Manag.

Online talk InterSci[1]

Science chat Grassmarkert[2]

Jury member FameLab[3]

Organizer AGEM[4] annual retreat

2020

Regional Finalist FameLab[3]

Organizer AGEM[4] annual retreat

2019

MSc Molecular Medicine
Erasmus MC

2 yrs

2015

Internship in the lab of Riccardo Fodde
Pathology Department, Erasmus MC
Role of cKit in the gut

10 mos

Organizer Dutch national student swimming annual competition

Board member and trainer Rotterdam swimming student team

1 yr

Internship in the lab of Derk ten Berg
Cell Biology Department, Erasmus MC
Gene therapy of Hurler's disease using mouse liver organoids

8 mos

Bachelor Biology
University of Coimbra, Portugal

3 yrs

2013

Organizer Portuguese national Biology students annual meeting

2012

Guide and children activities facilitator
Science Museum of Coimbra

6 mos

[1] Scientific conversations with lay audience
[2] Community Project for vulnerable adults, Edinburgh
[3] International science communication competition
[4] Amsterdam Gastroenterology Endocrinology Metabolism institute

REFERENCES

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Ingrid.RENES@danone.com

INTERESTS

Volunteering, reading, music, world politics, cooking, self-development

ACKNOWLEDGMENTS

The Tânia that entered the Tytgat Institute in January of 2016 was not the same Tânia that left in November of 2020. I learned so much about science and research, but also about myself. And for this, I am deeply thankful. In the next paragraphs, I mention the people that were part of my PhD journey and made it possible for me to be standing at Aula on the 10th June 2022.

Dear Vanesa, I still remember how impressed I was the first time I met you. And you did not disappoint. You became a true mentor for me. Despite our different personalities, we learned how to work together and I respect you immensely for that. Thank you for always bringing a new point of view, helping me see what really matters and believing in me. I could have not asked for a better supervisor.

Ingrid, I also remember very well our first contact. I had just arrived from visiting my family and was in the train from Schiphol to Rotterdam, when you called me to schedule an interview. Since then, and every time we speak, I get inspired by your energy, your optimism and your love for science. I think I do not know anyone else that has so much fun doing research! Thank you for challenging me, for the brainstorm sessions and for the motivation during the toughest moments. I will never forget.

Ruurd, one of the nicest memories is about the five of us going to the conference in Hannover. You drove us in your car and introduced us to the early life intestinal world. I really appreciate the time you took to meet one-on-one with me at the beginning. It helped me achieving this thesis and becoming a Doctor of Philosophy. Thank you for giving me the opportunity of a lifetime.

Doing research in the lab can be very lonely and isolating. But if you are part of a group like the DigeST team, you will never be alone or bored. I could write pages and pages of all the nice moments I spent with each one of you but I will try to keep it short. So here goes a tight hug to my scientific twin Marit, to the best officemates/lab experts/coffee break partners ever Jacqueline, Sander, Manon, and Paula, to the life of all parties and cell culture labs Florien, Jonathan, Bart, Wouter, Marileen, Ruben, Job, and Marte, to the expert and wise Manon and Pim, to the ones who left before me Olga, Bart Baan, Liset, and Gijs.

The true team spirit, however, goes further than the research group I belonged to. To the whole Tytgat Institute, thank you. You all made me feel welcome, from the discussions about everything and anything around the lunch table to the gezellig borrels. A special

shout out to the fellow members of the borrel committee and the AGEM retreat committee, definitely highlights of my PhD!

Thank you as well to the organoid aficionados of the AMC and to Joana and Ferhat that welcomed me at the NKI as if I was part of Liam's group.

Now, let us go to the other side of the story, the ones that kept me sane during these 6 years.

When I received the news that I was going to do a PhD at Tytgat, I called my family. But I also called Diana. She was in a meeting or a course, or something like that, and she left to pick up my call. She said she felt it was important and it was. She is always right. Diana, thank you for all your support and friendship, for listening to my rehearsal to each department presentation I gave, for being the amazing person that you are. I would not be who I am today without you.

Sometimes, a friend comes into your life unexpectedly. Jasmin and I met at the laboratory animal course at the Erasmus MC. We kept in touch afterwards and would meet sometimes. Then we found out that we really could understand each other and what we were going through during our own PhD life's. And our friendship started. Jasmin, thank you for all the fun moments and your support during the hardest days.

The fantastic trio of Rochusenstraat, Keane, Roberto, and Adrian, thank you for all the advices and laugh we always have. They gave me strength to never give up.

Sweet Mike, you were an important presence during the first times of my PhD that I will not forget. Your arms are always open for me and you showed me it is ok to be who I am. And thank you as well to my drum n bass friends for all the dancing and great music we witnessed together.

Maikel, thank you for always showing me the bright side and helping me believe I could do this.

And then, there are the bisons. I really believe there is no group like this anywhere else. The beautiful people in this group bring together a huge variety of cultures, spirits, believes, souls, vibes, and craziness. It is an honour to have met you!

Being away from family during such a challenging period of life is hard but I always felt the luckiest for having a family that would welcome me on the weekends, with whom

I could share my good and bad moments, with whom I could eat and drink and laugh, with whom I felt love. Thank you for everything Irma, Nico, Bas, Carrot, and Skip.

And now in Portuguese, for my family:

Querida família, obrigada por todo o vosso amor e força, por acreditarem em mim e aguentarem as saudades por eu estar longe. Esta tese é mais uma prova de que valeu a pena todo o esforço. Sou feliz e sei que isso é o mais importante para todos nós. Amo-vos!

No, I did not forget you. My dear sweetheart Francesca. For me, there is a pre-Francesca and a post-Francesca era at the Tytgat. Oh, how much happiness and joy you brought to our lab and to my life! It is an honor to have met you, I am a lucky cutie pie. I really don't think I would have been able to finish this PhD without you. And the best is that we now have a friendship for life <3 Thank you Francesca, from the bottom of my heart.

My schatje Alex, when I started this PhD I did not even know you. We managed to meet, fall in love, date, and marry in the meantime. No one endured the ups and downs of the PhD path as long and as intensely as you did. If I would need more proofs of your love, this would be a big one. Thank you for all the wise words and advices, for all the motivation you gave me, for all the celebrations of the small achievements in between, and for all the tears you cleaned away. You have a special way of completing me. Love you.

If my biggest fear came true and I forgot to mention your name, sorry.

With love, to you all,
Tânia G.

(Cheers!)