Developmental stage, solid food introduction and suckling cessation differentially influence the co-maturation of the gut microbiota and intestinal epithelium in rabbits

Martin Beaumont1*, Eloïse Mussard1, Céline Barilly1, Corinne Lencina1, Laure Gress1, Louise Painteaux1, Béatrice Gabinaud1, Laurent Cauquil1, Patrick Aymard1, Cécile Canlet2, Charlotte Paës1, Christelle Knudsen1, Sylvie Combes1

1 - GenPhySE, Université de Toulouse, INRAE, ENVT, F-31326, Castanet-Tolosan, France.
2 - Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRAE, ENVT, INP-Purpan, UPS, Toulouse, France

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*Corresponding author: Martin Beaumont, GenPhySE, Université de Toulouse, INRAE, ENVT, F-31326, Castanet-Tolosan, France. Telephone number: +33 05 61 28 51 85. E-mail: martin.beaumont@inrae.fr

Running title: Co-maturation of the gut microbiota and epithelium

Abbreviations: PND: postnatal day, PND18 Milk: group of exclusively suckling rabbits at PND18, PND25 Milk: group of exclusively suckling rabbits at PND25, PND25 Milk+Solid: group of suckling rabbits ingesting solid food at PND25, PND25 Solid: group of rabbits ingesting exclusively solid food at PND25, SCFA: short chain fatty acids, TLR: toll-like receptor.
ABSTRACT

Background: In mammals, the establishment around weaning of a symbiotic relationship between the gut microbiota and its host determines long-term health.

Objective: The aim of this study was to identify the factors driving the co-maturation of the gut microbiota and intestinal epithelium at the suckling-to-weaning transition. We hypothesized that developmental stage, solid food ingestion and suckling cessation contribute to this process.

Methods: From birth to day 18, Hyplus rabbits were exclusively suckling. From day 18 to day 25, rabbits were i) exclusively suckling or ii) suckling and ingesting solid food or iii) exclusively ingesting solid food. The microbiota (16S amplicon sequencing), metabolome (nuclear magnetic resonance) and epithelial gene expression (high-throughput qPCR) were analyzed in the caecum at day 18 and 25.

Results: The microbiota structure and metabolic activity were modified with age when rabbits remained exclusively suckling. The epithelial gene expression of nutrient transporters, proliferation markers and innate immune factors were also regulated with age (e.g. 1.5-fold decrease of TLR5). Solid food ingestion by suckling rabbits had a major effect on the gut microbiota by increasing its α-diversity, remodeling its structure (e.g. 6.3-fold increase of Ruminococcaceae) and metabolic activity (e.g. 4.6-fold increase of butyrate). Solid food introduction also regulated the gene expression of nutrient transporters, differentiation markers and innate immune factors in the epithelium (e.g. 3-fold increase of NOS2). Suckling cessation had no effect on the microbiota while it regulated the expression of genes involved in epithelial differentiation and immunoglobulin transport (e.g. 2.5-increase of PIGR).

Conclusion: In rabbits, the maturation of the microbiota at the suckling-to-weaning transition is driven by the introduction of solid food and to a lesser extent by developmental stage. In
contrast, the maturation of the intestinal epithelium at the suckling-to-weaning transition is under the influence of developmental stage, solid food introduction and suckling cessation.

**KEY WORDS**
Milk, weaning, bacteria, metabolites, development

**INTRODUCTION**
The microbiota colonizes the mammalian intestine at birth and its composition evolves with age towards an adult state, mainly under the influence of the diet. The gut microbiota is first shaped by components of maternal milk such as fat, proteins, oligosaccharides, immunoglobulins and antimicrobial peptides (1–3). Later in life, the introduction of solid food provides new plant-derived substrates for the microbiota, which results in the growth of bacteria able to utilize these nutrients (4,5).

The onset of solid food ingestion is also a critical step for the postnatal maturation of the intestinal epithelium. In the small intestine, an enzymatic switch occurs at the suckling-to-weaning transition with the decline of lactase involved in the digestion of milk lactose and the induction of disaccharidases involved in the terminal digestion of plant carbohydrates (6,7).

Key components of the epithelial defenses are also modulated around weaning, including tight-junctions, toll-like receptors (TLR), antimicrobial peptides and immunoglobulin transporters (4,8–12). The maturation of the epithelium at the suckling-to-weaning transition is partly driven by hormonal and genetically-wired factors (13–15). The timing of this developmental program governing epithelial maturation can be altered by modification of early life dietary intakes (6).

The gut microbiota also plays a pivotal role in the development of the intestinal epithelium at the onset of solid food ingestion (16–18). The microbial control of epithelial barrier
maturation involves various mechanisms, including activation of TLR signaling and production of bacterial metabolites such as butyrate (4,19). Moreover, bacterial signals can act on epithelial maturation at weaning in combination with host-derived factors such as glucocorticoids (20). Conversely, the maturation of host immunity and metabolism during weaning also shapes the gut microbiota, notably through the modulation of epithelial innate immune responses and bile acid secretions (21,22).

The co-maturation of the gut microbiota and intestinal epithelium at the suckling-to-weaning transition is thus orchestrated by developmental signals and dietary intakes. Deciphering the relative contribution of these endogenous and nutritional factors has major implications for health since the establishment of a symbiotic relationship between the microbiota and its host during a time-window around weaning determines the long-term susceptibility to immune and metabolic diseases (23,24). We hypothesized that developmental stage, solid food ingestion and suckling cessation contribute to drive the co-maturation of the gut microbiota and intestinal epithelium at the suckling-to-weaning transition. Our objective was to decipher their respective contribution to this process.

Controlling early life dietary intakes is a technical challenge in rodent models because maternal separation causes stress that disrupts intestinal homeostasis (25). Thus, the rabbit was selected as a model to study the co-maturation of the gut microbiota and epithelium in mammals. Indeed, in this species, suckling occurs once a day for 5 minutes. After that, the mother leaves the nest and the pups are thus separated from the mother for the rest of the day (26). In experimental conditions, this characteristic allows a precise control of milk and solid food ingestion by respectively weighing the dam before and after suckling and the pups feeders, that the dams cannot access, thus solely measuring the pups intake. Therefore, the rabbit model allowed us to accurately control milk and solid food availability at the suckling-to-weaning transition. We studied the development of the microbiota and epithelium in the
caecum between postnatal day (PND) 18, which corresponds to the spontaneous onset of significant solid food ingestion, and PND25 in rabbits that were i) exclusively suckling or ii) suckling and ingesting solid food or iii) exclusively ingesting solid food. The caecum was studied since this digestive segment is an important site of interaction between the microbiota and the digestive epithelium in rabbits (4). Analysis of microbiota composition, metabolome and epithelial gene expression highlighted the differential influence of developmental stage, solid food introduction and suckling cessation on the co-maturation of the microbiota and epithelium at the suckling-to-weaning transition.

MATERIAL AND METHODS

Animal experiments

The experiment was performed at the INRAE PECTOUL experimental facility (GenPhySE, INRAE, Castanet-Tolosan, France). Animals were handled according to the European Union recommendations on the protection of animals used for scientific purpose (2010/63/EU) and in agreement with the French legislation (NOR: AGRG1238753A 2013). This protocol received the approval of the local ethics committee (SSA_2019_004). The rabbits studied were crossbreeds from two commercial lines (maternal line: Hyplus PS19, paternal line: Hyplus PS59; Hypharm, France). Multiparous dams (n=8) were housed individually in wire cages (61 x 69 x 49 cm) equipped with a closed nest for the pups (39 x 27 x 35 cm). The litter size was standardized to ten pups per litter by cross-fostering at PND2.

A schematic representation of the experimental design is presented in supplementary figure 1. All rabbit pups were exclusively suckling from birth to PND18. From this day onward, rabbits were divided into three groups (figure 1A). In the first group (PND25 Milk, n=4 litters, 1 litter/cage), as pups were exclusively suckling until PND25, the litter size was reduced to 5 pups/litter to ensure an adequate supply of milk. In the second group (PND25
Milk+Solid, n=4 litters, 8 pups/litter, 1 litter/cage), pups were suckling and had *ad libitum* access to solid food pellets (StabiPro, Terrya, Rignac, France). In the third group, rabbit pups (PND25 Solid, n=12 pups from 4 litters in 2 cages) were weaned at PND18 and had *ad libitum* access to solid food pellets (StabiPro) but not to maternal milk. The chemical composition of the solid diet is shown in *supplementary table 1*. For suckling, the dam was placed once a day (5 min) in the nest of the pups. Milk ingestion by the litter was quantified at PND17 and 24 by weighing the dam before and after suckling. Immediately after suckling, feces from the dam were removed from the nest to prevent coprophagia by the pups. Litter solid food intake was quantified between PND21 and 24 by weighing the feeder. The dam had no access to the feeder of the pups and vice versa.

**Sample collection**

Rabbit pups were euthanized by electronarcosis followed by exsanguination (supplementary figure 1). At PND18, 2 pups/litter (8 litters, n=16, 8 females and 8 males) were euthanized after suckling (PND18 Milk). In the group of exclusively suckling rabbits (PND25 Milk), 3 pups/litter (4 litters, n=12, 8 females and 4 males) were euthanized at PND25 after suckling. In the group of suckling rabbits with access to solid food pellets (PND25 Milk+Solid), 3 pups/litter (4 litters, n=12, 6 females and 6 males) were euthanized at PND25 after suckling. In the group of early weaned rabbits (PND25 Solid, n=12, 8 females and 4 males), all pups were euthanized at PND25. The caecum was isolated and weighted. Caecal content was collected and kept on ice until transfer to -80°C for long-term storage. Caecal content pH was measured with a glass electrode (VWR Collection SP225, Radnor, PA). One g of caecal content was placed in 1 mL of 2% (w/v) H₂SO₄ for ammonia (NH₄⁺) quantification with a colorimetric method using a continuous flow analyzer SAN++ (Skalar, Norcross, GA) as described previously (27). NH₄⁺ and pH measurements could not be performed in some
samples due to insufficient amount of caecal content. For histological analyses, a section of caecal tissue with content was collected and placed in Carnoy’s fixative solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 3 hours before transfer in 70% ethanol and stored at 4°C. Caecal tissue fragments (4 x 2 cm) were washed in cold PBS and stored on ice until epithelial cell isolation.

**16S rRNA gene amplicon sequencing and sequence analysis**

Caecal content DNA was extracted using the Quick-DNA Fecal/Soil Microbe 96 Kit (ZymoResearch, Irvine, CA) and the 16S rRNA gene V3-V4 region was amplified by PCR and sequenced by MiSeq Illumina Sequencing as previously described (28). Sequencing reads were deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA accession: PRJNA699723). 16S rRNA gene amplicon sequences were analyzed using the FROGS pipeline according to standard operating procedures (29). Amplicons were filtered according to their size (300-500 nucleotides) and clustered into OTUs using Swarm (aggregation distance: d=1 + d=3). After chimera removal, OTUs were kept when present in at least 3 samples and representing more than 0.005% of the total number of sequences (30). OTUs affiliation was performed using the reference database silva138 16S with a minimum pintail quality of 100 (31). The mean number of reads per sample after filtering was 20 131 (min: 11 045 - max: 37 579).

**NMR metabolomics**

The metabolome was analyzed in caecal content (50 mg) as described previously by using nuclear magnetic resonance (NMR) at the MetaboHUB-MetaToul-AXIOM metabolomics platform, Toulouse, France (4). The results are presented as relative concentrations, with the PND18 Milk group used as a reference. Annotated representative spectra in each group are
presented in supplementary figure 2. For each of the 29 identified metabolite, buckets non-overlapping with other metabolites were selected for the quantification (supplementary table 2).

Caecal epithelial cell isolation

Caecal tissue fragments stored in cold PBS were transferred in 5 mL of cold dissociation solution (9 mM EDTA [Thermo Fisher Scientific, Waltham, MA], 3 mM 1,4-Dithiothreitol [DTT, Roche, Basel, Switzerland] in PBS without Ca\(^{2+}\)/Mg\(^{2+}\) [Thermo Fisher Scientific, Waltham, MA]). After incubation (30 min, room temperature, under agitation), caecal tissue was transferred in 5 mL cold PBS without Ca\(^{2+}\) and Mg\(^{2+}\). Epithelial crypts were detached by vigorous manual shaking (1 min). After removal of the remaining caecal tissue, the presence of epithelial crypts in the solution was verified by brightfield microscopy (supplementary figure 3). After centrifugation (300 g, 5 min, 4°C), the supernatant was discarded and the crypt pellet was resuspended by vortexing in 300 µL cold TRI Reagent (ZymoResearch, Irvine, CA) before storage at -80°C until RNA extraction.

Gene expression profiling in epithelial cells

After thawing, epithelial crypts lyzed by pipetting in TRI Reagent were vortexed before centrifugation (12 000 g, 4°C, 10 min) to remove particles and the supernatant was used for total RNA purification by using the Direct-zol RNA MiniPrep Plus kit (ZymoResearch, Irvine, CA) following the manufacturer’s instructions, including a DNAse I treatment. RNA concentration and purity were analyzed with a NanoDrop 8000 (Thermo Fisher Scientific, Waltham, MA). cDNA were prepared from 500 ng RNA with the GoScript™ Reverse Transcription Mix, Random Primers kit (Promega, Madison, WI) following the manufacturer’s instructions. High throughput real-time qPCR was performed using the
Biomark microfluidic system using a 96.96 Dynamic Array™ IFC for gene expression (Fluidigm, San Francisco, CA) according to the manufacturer’s recommendations. The sequences of the primers used are presented in supplementary table 3. Data were analyzed with the $2^{-\Delta \Delta Ct}$ method with GAPDH gene expression used as a reference (32). The PND18 Milk group was used a reference for normalization.

**Histology**

Transversal sections of caecal tissue with luminal content fixed in Carnoy’s solution were embedded in paraffin and stained by hematoxylin-and-eosin in the histology platform Genotoul Anexplo (Toulouse, France). Slides were digitalized and crypt depth (>8 well-oriented crypts/sample) was measured with the CaseViewer 2.3 software (3DHISTECH, Budapest, Hungary). The measurement was repeated twice by two independent investigators blinded to the groups.

**Immunoglobulin A quantification**

Caecal content was diluted at 50 mg.mL$^{-1}$ in TBS buffer. After shaking thoroughly, the samples were centrifuged (3000 g, 10 min, 4°C). The supernatants were collected and stored at -20°C until analysis. The total caecal IgA contents were determined in duplicates by sandwich ELISA by using polyclonal goat anti-rabbit IgA antibodies (cat# A120-109P and A120-109A, Bethyl Laboratories, Montgomery, TX). Sample dilution was adapted to experimental groups (suckling rabbits 1:1280 - 1:5120, weaned rabbits 1:320). Absorbance was measured at 450 nm with a GloMax Discover plate reader (Promega, Madison, WI). Relative IgA concentrations were calculated by using a standard curve obtained by a serial dilution of a pool of all samples (rabbit IgA standard is not commercially available). IgA relative concentrations were normalized to the protein concentration in the caecal content.
measured with a colorimetric assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Biorad, Hercules, CA).

**Statistical analysis**

All statistical analyses were performed using the R software (version 4.0.3). The microbiota composition analysis was performed using the phyloseq package (version 1.26.1) (33). For α and β diversity analyses, the samples were rarefied to even sequencing depth (11045 reads per sample). Richness (observed OTUs) and Shannon α-diversity were calculated. The β-diversity was analyzed using the Bray-Curtis distance and plotted by non-Metric Dimensional Scaling (nMDS). Permutational Multivariate Analysis of Variance (PERMANOVA) was used to test the effect of groups on the Bray-Curtis distance between samples by using the vegan package (version 2.5-7). For differential abundance analysis, OTUs representing less than 0.05% of the total number of sequences were filtered out. OTUs unrarefied counts were agglomerated at phylum, family or genus level and relative abundances were calculated at each taxonomic level. Principal component analyses (PCA) were performed with the mixOmics package on data obtained for metabolomics (relative abundance of 29 metabolites) and gene expression profiling (relative expression of 50 genes) (version 6.14.0) (34). The heatmap representation was created with the pheatmap package (version 1.0.12) using Euclidean distance and Ward algorithm to cluster genes according to their relative expression.

Univariate statistical analyses to compare the four groups were performed with non-parametric Kruskal-Wallis tests and the obtained P-values were adjusted by the Benjamini & Hochberg method for bacterial groups (family and genus level) and metabolites. When the overall group effect was significant (P<0.05), groups were compared pairwise using Wilcoxon tests with the Holm correction. No effect of sex was observed on any variate, and was thus not taken into account in statistical analyses. The effects of age were identified by
comparison of exclusively suckling rabbits at PND18 and PND25 (PND18 Milk versus PND25 Milk, figure 1A). The effects of solid food introduction were identified by comparison at PND25 of exclusively suckling rabbits with rabbits that ingested milk and solid food (PND25 Milk versus PND25 Milk+Solid). The effects of suckling cessation were identified by comparison at PND25 of rabbits that ingested milk and solid food with rabbits that ingested exclusively solid food (PND25 Milk+Solid versus PND25 Solid).

RESULTS

Milk ingestion level was similar at PND18 and 25 in suckling rabbits consuming or not solid food (mean: 35 g.pup⁻¹.day⁻¹, n=8 litters at PND18 and n=4 litters per group at PND25, supplementary figure 4A). At PND25, solid food ingestion was numerically 3-fold higher in rabbits consuming solid food only (PND25 Solid, n=2 cages of 6 rabbits) when compared to rabbits consuming milk and solid food (PND25 Milk + Solid, n=4 litters) (19.5 and 6.5 g.pup⁻¹.day⁻¹, respectively, supplementary figure 4B). When rabbits ingested both milk and solid food at PND25, solid food represented only 17% of the total fresh matter intake. The body weight of rabbits increased between PND18 and 25, in all groups (supplementary figure 4C). The relative caecum weight increased between PND18 and 25 only when rabbits ingested solid food (figure 1B) and, as expected, plant-derived residues were observed in the caecal content only in these animals (figure 1C).

Influence of age and dietary intakes on the caecal microbiota diversity and structure

The relative abundance of each bacterial phylum, family and genus are presented in supplementary tables 4-6. β-diversity analysis at the OTU level using the Bray-Curtis distance indicated that the microbiota structure was modified with age in exclusively suckling rabbits (PND18 Milk vs PND25 Milk, explained variance R²=0.21, P<0.001) (figure 2B).
The relative abundances of Bacteroidota and families classified into this phylum (Rikenellaceae, Barnesiellaceae, Marinifilaceae) and Campylobacterota decreased with age while the relative abundance of Firmicutes and families classified into this phylum (Oscillospiraceae, Anaerovoracaceae, Christensenellaceae) increased with age (figure 2C-E, supplementary table 4). At PDN25, solid food ingestion by suckling rabbits increased OTU richness and Shannon α-diversity and strongly modified the microbiota structure (PND25 Milk vs PND25 Milk+Solid, explained variance R²=0.26, P<0.001) (figure 2A and B). The introduction of solid food prevented the age-related increase of Oscillospiraceae, Anaerovoracaceae and the age-related decrease of Barnesiellaceae (figure 2C and D). In contrast, solid food introduction amplified the increased relative abundance of Christensenellaceae observed with age (figure 2D). The introduction of solid food also increased the relative abundance of Ruminococcaceae and Monoglobaceae while it reduced the relative abundance of Enterobacteriaceae and Eggerthellaceae (figure 2D and E). Strikingly, suckling cessation in rabbits ingesting solid food had no effect on the microbiota diversity and structure (PND25 Milk+Solid vs PND25 Solid). Altogether, our results indicate that the microbiota was mainly influenced by solid food introduction and to a lesser extent by age while suckling cessation had no effect.

Influence of age and dietary intakes on the caecal metabolome

PCA indicated that the caecal metabolome was mainly influenced by the introduction of solid food (PCA axis 1, 45% of variance) and by age (PCA axis 2, 11% of variance) (figure 3A, supplementary table 7). The relative concentration of acetate, butyrate, propionate, glucose and glutamate increased with age in exclusively suckling rabbits while the relative concentration of 3-methyl-2-oxobutyrate, 4-methyl-2-oxovalerate, 3-phenylpropionate, trimethylamine and dimethylamine decreased with age (PND18 Milk vs PND25 Milk, figure
Solid food ingestion by suckling rabbits amplified the age-related increase of acetate, butyrate and glucose and the age-related decrease of 4-methyl-2-oxovalerate (PND25 Milk vs PND25 Milk+Solid, figure 3B and D). In contrast, solid food introduction reduced the age-related increase of propionate (figure 3B). Solid food introduction had the opposite effect of age on 3-phenylpropionate since it strongly increased its relative concentration (figure 3D). The introduction of solid food also decreased the caecal pH and the relative concentrations of succinate, formate, choline, ammonia and amino acids (figure 3C-G). Suckling cessation in rabbits ingesting solid food reduced the relative concentration of propionate, 4-methyl-2-oxovalerate and methylvamine while it increased the relative concentration of 3-phenylpropionate (PND25 Milk+Solid vs PND25 Solid, figure 3A, D and E). Taken together, our metabolomics data indicated that the changes of the gut microbiota metabolic activity at the suckling-to-weaning transition was mainly driven by solid food introduction and to a lesser extent by age and by suckling cessation.

Influence of age and dietary intakes on gene expression in the caecal epithelium

PCA revealed that gene expression in the caecal epithelium was influenced by age, solid food introduction and suckling cessation (figure 4A). We identified a first cluster of genes (Cluster 1) which expression increased with a cumulative effect of age, solid food introduction and suckling cessation (figure 4B). This cluster included genes coding for epithelial differentiation markers (KRT20, CA2, AQP8, ALPI), mediators of epithelial defenses (LYZ, PIGR, NOS2, GPX2) and nutrient transporters (SLC16A1, SLC38A3). In contrast, we identified a second cluster of genes (Cluster 2) which expression decreased with a cumulative effect of age, solid food introduction and suckling cessation (figure 4B). This cluster included genes coding for epithelial stem cell markers (LGR5, SOX9), mediators of epithelial innate immunity (REG3G,
DEFB1, TLR5, GPX1), epithelial barrier components (MUC1, MUC13, CLDN1, CLDN2) and amino acid transporters (SLC6A19, SLC15A1, SLC16A10, SLC38A5).

Epithelial transport of nutrients

The gene expression of peptide and amino acid transporters (SLC15A1, SLC6A19, SLC38A5, SLC7A7, SLC16A10) was downregulated with age in exclusively suckling rabbits (PND18 Milk vs PND25 Milk, figure 5A-C). Ingestion of solid food by suckling rabbits amplified the downregulation of the peptide transporter SLC15A1 while it upregulated the expression of transporters for amino acids SLC38A3 and short chain fatty acids (SCFA) (SLC16A1) (PND25 Milk vs PND25 Milk+Solid, figure 5A-C). Suckling cessation in rabbits ingesting solid food amplified the downregulation of amino acid transporters (SLC6A19 and SLC16A10) and the upregulation of the SCFA transporter SLC16A1 (PND25 Milk+Solid vs PND25 Solid, Figure 5B and C).

Epithelial renewal and differentiation

The gene expression of the stem cell marker LGR5 and of HES1 (a transcription factor repressing secretory lineage commitment) were reduced with age in exclusively suckling rabbits while the gene expression of absorptive cell markers (ALPI and CA2) increased with age (PND18 Milk vs PND25 Milk, figure 6A-D). Ingestion of solid food by suckling rabbits reduced the expression of transmembrane mucins (MUC1 and MUC13) while it upregulated the gene expression of the differentiation maker KRT20 and of KLF4, a transcription factor involved in goblet cell differentiation (PND25 Milk vs PND25 Milk+Solid, figure 6C and D). Suckling cessation in rabbits ingesting solid food amplified the downregulation of LGR5 and MUC1 and the upregulation of ALPI and KRT20 (PND25 Milk+Solid vs PND25 Solid, figure 6A, C and D). Suckling cessation also downregulated the expression of the proliferation
marker PCNA and upregulated the expression of the absorptive cell marker AQP8 (figure 6A and C). Epithelial crypt depth increased between PND18 and PND25 when rabbits ingested either only solid food or only milk but surprisingly not when ingesting solid food and milk (figure 6B).

*Epithelial barrier*

The expression of the polymeric immunoglobulin receptor (PIGR) increased with age in exclusively suckling rabbits while the expression of the antimicrobial peptide REG3G, the Toll-like receptor 5 (TLR5) and the tight junction proteins claudin 1 and 2 (CLDN1 and CLDN2) decreased with age (PND18 Milk vs PND25 Milk, figure 7A-C and E). Ingestion of solid food by suckling rabbits amplified the down regulation of CLDN1 (PND25 Milk vs PND25 Milk+Solid, figure 7E). The introduction of solid food also upregulated the gene expression of the cytokine interleukin-18 (IL18), the pro-oxidant enzyme inducible nitric oxide synthase (NOS2) and the anti-oxidant enzyme glutathione peroxidase 2 (GPX2) while it downregulated the expression of the antimicrobial peptides lysozyme (LYZ) and defensin beta 1 (DEFB1) (figure 7B-D). Suckling cessation in rabbits ingesting solid food amplified the upregulation of PIGR and this effect was associated with a strong reduction of IgA concentration in the caecum (PND25 Milk+Solid vs PND 25Solid, figure 7A). Suckling cessation also amplified the downregulation of REG3G and TLR5 while it strongly upregulated the gene expression of LYZ (figure 7B and C). Overall, gene expression profiling indicate that the maturation of the intestinal epithelium at the suckling-to-weaning transition was influenced by the combination of age, solid food introduction and suckling cessation.
DISCUSSION

The suckling-to-weaning transition is considered to be a window of opportunity for the development of a mutualistic relationship between the gut microbiota and its host with long term consequences for health (23,24). Indeed, the gut microbiota composition and metabolic activity are highly remodeled at the onset of solid food ingestion which coincides with the maturation of the intestinal epithelium (4,9,35). However, deciphering the relative contributions of endogenous and dietary factors driving this developmental process is an experimental challenge. Here, we took advantage of the unique suckling behavior of rabbits (mother-pup separation except for 5 min once a day) to delineate the influence of developmental stage (age), solid food introduction and suckling cessation on the co-maturation of the gut microbiota and epithelium at the suckling-to-weaning transition.

Influence of age in exclusively suckling rabbits

The slight modification of the microbiota structure with age in exclusively suckling rabbits is in agreement with a previous study using the same model (36). By using culture-based methods, the authors observed a reduction of the colibacilli flora with age in the caecum although only maternal milk was ingested (36). The age-related regulation of epithelial innate immunity observed in our study (e.g. downregulation of the gene expression of REG3G and TLR5) could contribute to the modification of the microbiota composition with age in exclusively suckling rabbits. Indeed, the age-dependent modification of TLR5 expression was shown in mice to influence the microbiota composition through the antimicrobial peptide REG3G (22). Changes in milk composition according to lactation stage (PND18 vs 25) could also influence the gut microbiota. For instance, the milk oligosaccharides composition changes during lactation in humans (37). However, the variation of milk oligosaccharides
composition according to lactation stage has not been described in rabbits. Importantly, developmental stage did not modify the microbiota towards a solid-food oriented microbiota that can be considered as more mature. The age-related increase in concentration of the bacterial metabolites acetate, propionate and butyrate could be related to an increased availability for the microbiota of substrates that can be metabolized into SCFA, either derived from milk (e.g. oligosaccharides) or from the host (38). The important increase with age of propionate (4-fold) is in agreement with previous results obtained in exclusively suckling rabbits (36).

The age-related decline in expression of amino acid transporters in the epithelium is consistent with previous studies showing a gradual reduction of amino acid transport from birth to weaning in mammals, including rabbits (39). This effect was observed despite the absence of change in the relative concentration of amino acids in the lumen, suggesting that the regulation of these transporters is driven by an intrinsic developmental program corresponding to the capacity of the large intestine to absorb peptides and amino acids mostly in early life (40). Indeed, ontogenic factors (e.g. hormonal status) are thought to play an important role in the maturation of the intestinal epithelium (6,7). Endogenous factors could also be involved in the upregulation of PIGR expression that we observed with age in exclusively suckling rabbits, independently of modifications of caecal IgA concentrations. Previous studies in mice demonstrated that the upregulation of PIGR at weaning is driven by hormonal modifications during this developmental transition (41). The downregulation of the gene expression of the epithelial stem cell marker LGR5 with age in exclusively suckling rabbits could be linked to a decreased concentration of milk growth factors, as observed before weaning in mice (42). Our results also showed that developmental factors play a major role in the decline of the gene expression of epithelial junction proteins (CLDN1 and CLDN2) observed at the suckling-to-weaning transition in rabbits (4). These results probably reflect the
important synthesis of tight junction proteins in the developing caecum that gradually decreases as maturation progresses.

Altogether, our results show that age influences the gut microbiota and gene expression in the epithelium. Ontogenic factors (e.g. hormonal status) and modification of milk composition according to lactation stage (e.g. oligosaccharides, growth hormones) probably play an important role in these effects.

Influence of solid food ingestion in suckling rabbits

The major effect of solid food introduction on the gut microbiota richness (~1.5-fold increase) and structure can be linked to the presence in the caecum content of new substrates derived from plants. In agreement with this hypothesis, solid food introduction induced a bloom in Ruminococcaceae (10-fold increase) which is a bacterial family specialized in complex plant polysaccharides degradation and that typically blooms after weaning in humans, pigs and rabbits (4,43–45). Primary degradation of plant carbohydrates by the microbiota probably explains the increased concentration of glucose observed in the caecum after the introduction of solid food (46). Subsequent glucose fermentation by the microbiota might explain the increased concentration in SCFA induced by solid food introduction as observed in previous studies (4,46–48). This increased production of SCFA by the microbiota could be responsible for the increased gene expression of the epithelial transporter of SCFA (SLC16A1/MCT1) since its expression is upregulated by butyrate (49). The increased concentration of butyrate could also contribute to the pro-differentiation effect of the introduction of solid food (e.g. upregulation of KRT20 expression) since this bacterial metabolite is known to promote epithelial differentiation (50).

The increased concentration of the polyphenol-derived bacterial metabolite 3-phenylpropionate (hydrocinnamic acid) upon introduction of solid food suggested again a
regulation of the metabolic activity of the microbiota by plant derived substrates (51). In contrast, the reduction in the microbial amino acid catabolic product ammonia after the introduction of solid food can be linked to the low concentration of amino acids in the caecal lumen (52).

The remodeling of the microbiota composition induced by the introduction of solid food probably triggers the upregulation of the gene expression of the cytokine IL18 and of the enzymes involved in epithelial redox homeostasis NOS2 and GPX2. Indeed, the colonization of the gut by the microbiota is required for the upregulation of NOS2 at weaning in mice (17). In turn, this upregulation of epithelial innate immune responses could also shape the microbiota composition (53). Importantly, the major influence of solid food introduction on the microbiota and epithelium was observed despite the quantity of solid food ingested was much lower that the quantity of milk ingested. This observation suggests that the introduction of a small amount of solid food is sufficient to trigger the co-maturation of the microbiota and epithelium in suckling mammals.

Influence of suckling cessation in rabbits ingesting solid food

The absence of effects of suckling cessation on the microbiota diversity and structure suggests that once solid food has been ingested, it overwhelms the influence of milk components that are known to shape the microbiota (e.g. oligosaccharides, IgA, innate immune factors) (1,3). In contrast with our results, longitudinal studies in humans suggested that the maturation of the gut microbiota was driven by cessation of breastfeeding rather than by the introduction of solid foods (54,55). However, in these human studies, the effects of the cessation of breastfeeding and solid food introduction cannot be completely disentangled due to gradual dietary transitions (5). In comparison to the microbiota, the effects of suckling cessation were more pronounced at the epithelial level. The downregulation of stem cell and proliferation
markers after suckling cessation is consistent with a role for milk growth hormones in epithelial proliferation (6). The loss of milk hormones could also contribute to the strong pro-differentiation effect of suckling cessation. Alternatively, the stress potentially caused by the early weaning procedure could result in an increased glucocorticoid level, which is known to enhance epithelial differentiation (7). The strong upregulation of PIGR expression induced by suckling cessation could be due to the loss of maternal transfer of immunoglobulins (i.e. passive immunity), as demonstrated in mouse studies (10). Indeed, very low IgA levels were measured in rabbit caecum after suckling cessation, which is consistent with the maternal origin of IgA before weaning (i.e. passive immunity) (56). Interestingly, most of the effects of suckling cessation at the epithelial level corresponded to an amplification of the effect of age. This observation is consistent with the idea that dietary modification can accelerate but not override the genetically-wired development of the epithelium (6,7). Altogether, our results suggest that the loss of milk-derived molecules (e.g. growth hormones, IgA) after suckling cessation has no effect on the microbiota while it probably accounts for the major part of the effect on the epithelium development.

Conclusion

Our results show that the gut microbiota maturation at the suckling-to-weaning transition is mainly driven by the introduction of solid food and to a lesser extent by age while suckling cessation had no effect. Solid food introduction was also the main factor influencing the production of metabolites by the microbiota while the influence of age and suckling cessation were lower. Complex interactions between developmental stage, solid food introduction and suckling cessation were involved in the maturation of the intestinal epithelium. Additional studies are required to determine the optimal timing of solid food introduction and its
nutritional composition in order to promote the co-maturation of the gut microbiota and epithelium with the aim to promote long-term health (57).

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Statement of authors’ contributions to manuscript.

MB, CP and SC designed research; MB, EM, CB, CL, LG, LP, BG, PA, CC and CK conducted research; MB, LC and SC analyzed data; and MB and SC wrote the paper. MB had primary responsibility for final content. All authors have read and approved the final manuscript.

Data sharing

Data described in the manuscript will be made available upon request. Sequencing reads were deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA accession: PRJNA699723).
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Figure 1: Influence of age and dietary intakes on rabbit caecum development. A - Experimental design. B - Caecum weight (tissue + content), expressed as a percentage of the live body weight. Barplots show mean values and standard error of the mean. The overall effect of experimental groups was tested with a Kruskal-Wallis test. Groups were compared pairwise with Wilcoxon tests. Labeled means without a common letter differ, P<0.05. PND18 Milk: n=16, PND25 Milk: n=12, PND25 Milk+Solid: n=12, PND25 Solid: n=12. C - H&E stained caecal sections observed at low (upper row, red scale bars represent 1 mm) or high (lower row, black scale bars represent 0.1 mm) magnification. Yellow arrows indicate plant residues in the caecal content.
Figure 2: Influence of age and dietary intakes on rabbit caecum microbiota. A - Microbiota richness and Shannon α-diversity index. B - Non Metric Dimensional Scaling
(nMDS) two-dimensional representation of the microbiota β-diversity using Bray Curtis distance calculation (stress=0.17). C to E - Relative abundance of bacterial families classified within the Bacteroidota phylum (C), Firmicutes phylum (D) and other lower abundance phyla (E). A, C to E - Barplots show mean values and standard error of the mean. The overall effect of experimental groups was tested with a Kruskal-Wallis test. Groups were compared pairwise with Wilcoxon tests. Labeled means without a common letter differ, P<0.05. PND18 Milk: n=16, PND25 Milk: n=12, PND25 Milk+Solid: n=12, PND25 Solid: n=12.
Figure 3: Influence of age and dietary intakes on rabbit caecum metabolome. A: Individual plot of principal component analysis (PCA) of metabolites. B - Relative concentration of short chain fatty acids. C: Caecal content pH. The measure was not
performed at PND18 due to insufficient amount of caecal content. D - Relative concentration of miscellaneous metabolites. E - Relative concentration of choline and of its microbial catabolites. F: Ammonia (NH$_4^+$) concentration in caecal content. The measure was not performed at PND18 due to insufficient amount of caecal content. G - Relative concentration of amino acids. B to G - Barplots show mean values and standard error of the mean. The overall effect of experimental groups was tested with a Kruskal-Wallis test. Groups were compared pairwise with Wilcoxon tests. Labeled means without a common letter differ, P<0.05. PND18 Milk: n=16, PND25 Milk: n=12, PND25 Milk+Solid: n=12, PND25 Solid: n=12.
Figure 4: Influence of age and dietary intakes on gene expression in rabbit caecum. A: Individual plot of principal component analysis (PCA) of gene expression. B: Heatmap representing the relative expression of genes (columns) in individual samples (rows). The
colors represent the Z-scores (row-scaled relative concentration) from low (blue) to high values (red). Genes (columns) were clustered by the Ward method. PND18 Milk: n=16, PND25 Milk: n=12, PND25 Milk+Solid: n=12, PND25 Solid: n=12.
Figure 5: Influence of age and dietary intakes on epithelial nutrient transport in rabbit caecum. 

A - Schematic representation of the cellular localization of nutrient transporter in intestinal epithelial cells (Created with BioRender.com). Relative mRNA level of apical (B) and basolateral (C) nutrient transporters. Barplots show mean values and standard error of the mean. The overall effect of experimental groups was tested with a Kruskal-Wallis test.
Groups were compared pairwise with Wilcoxon tests. Labeled means without a common letter differ, P<0.05. PND18 Milk: n=16, PND25 Milk: n=12, PND25 Milk+Solid: n=12, PND25 Solid: n=12.
Figure 6: Influence of age and dietary intakes on epithelium renewal and differentiation in rabbit caecum. A - Relative mRNA level of stem cell and proliferation markers. B - Caecal epithelial crypt depth. C - Relative mRNA level of epithelial differentiation and absorptive cell markers. D - Relative mRNA level of secretory cell (goblet and enteroendocrine) markers. A - D - Barplots show mean values and standard error of the mean.
The overall effect of experimental groups was tested with a Kruskal-Wallis test. Groups were compared pairwise with Wilcoxon tests. Labeled means without a common letter differ, P<0.05. PND18 Milk: n=16, PND25 Milk: n=12, PND25 Milk+Solid: n=12, PND25 Solid: n=12.
Figure 7: Influence of age and dietary intakes on epithelial barrier function in rabbit caecum. A - Relative mRNA level of the polymeric immunoglobulin transporter.

Immunoglobulin A was quantified in caecal content by ELISA. AU: arbitrary units. The results were normalized by the protein concentration in the caecal content. B to E - Relative
mRNA level of antimicrobial peptides (B), proteins involved in microbial sensing (C), redox homeostasis (D) and epithelial junctions (E). A to E - Barplots show mean values and standard error of the mean. The overall effect of experimental groups was tested with a Kruskal-Wallis test. Groups were compared pairwise with Wilcoxon tests. Labeled means without a common letter differ, P<0.05. PND18 Milk: n=16, PND25 Milk: n=12, PND25 Milk+Solid: n=12, PND25 Solid: n=12.