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This protocol is part of the project METABOWEAN (Gut microbiota-derived metabolites: natural products to promote epithelial barrier maturation at the suckling-to-weaning transition) funded by the French National Research Agency (ANR-21-CE20-0048).

Objective of the protocol: The aim of this protocol was to use single cell transcriptomics to identify how the ingestion of solid food alters gene expression in each cell type of the caecum epithelium in suckling rabbits.

Animal experiments

The experiment was performed at the PECTOUL experimental facility (GenPhySE, INRAE, Toulouse, France). The handling of rabbits followed the recommendations outlined in the European Union's regulations for the protection of animals used in scientific research (2010/63/EU), and was consistent with French legislation (NOR: AGRG1238753A 2013). This protocol received approval from the local ethics committee "Comité d'éthique en expérimentation animale SCIENCE ET SANTE ANIMALES" N◦115 (SSA_2022_012). Multiparous dams (n = 4) were housed individually in wire cages (61 x 68 x 50 cm) equipped with a closed nest (39 × 27 × 50 cm). The litter size was limited to 10 pups per litter. From postnatal day (PND) 4, pups were placed in a new cage, separated from their mother. At PND12, litters were equalized to 6 pups in order to maximize milk ingestion. Pups from each litter were separated in 2 cages (3 pups/cage) to form two groups (Figure 1). In the first group (Milk), the pups were exclusively suckling. In the second group (Milk+Solid), the pups were suckling while having ad libitum access to food pellets (StabiGreen, Terrya). During all the experiment, the dam and the pups were placed once a day for 5-10 minutes in the nest for suckling before returning to their respective cages. Coprophagia was prevented by removing feces in the nest after suckling.

Sample collection

One male pup per litter (n=4) and per group (Milk or Milk+Solid) was sacrificed after suckling at PND24 or PND25 by electronarcosis followed by exsanguination. A fragment of caecal tissue was collected and placed in cold PBS without Ca^{2+}/Mg^{2+} (ThermoFisher scientific, cat#10010-015) until cell isolation.

Caecal epithelial cell isolation

Caecal tissue was opened longitudinally and washed with cold PBS to remove all contents. The tissue was minced into 1 cm^2 sections and washed with cold PBS. Tissue segments were transferred to 5 mL of a pre-warmed (37°C) digestion solution prepared in HBSS without Ca^{2+}/Mg^{2+} (ThermoFisher Scientific, cat#14175095) and supplemented with 5 mM EDTA (ThermoFisher Scientific, cat#AM9260G) and 1 mM DTT (Sigma, cat# 10197777001). After incubation (20 minutes at 37°C under slow agitation at 15 rpm), epithelial crypts were detached by vigorous shaking manually for one minute. The crypt solution was then filtered (100 µm) before centrifugation (300 x g for 5

minutes at 4°C). The crypt pellet was resuspended in 10 mL of pre-warmed dissociation solution containing TrypLE (ThermoFisher, cat# 1205036) supplemented with 1 mg/mL DNAse I (Sigma, cat # 10104159001), 5 mM $MgCl_2$ (Sigma, cat# M1028), 10 µM Y27632 (StemCell Technologies, cat# 72304) and the solution was distributed in two 50 mL conical tubes (5 mL/tube). Cells were incubated for 10 minutes at 37°C with gentle agitation at 15 rpm before homogenization by vortexing (3 seconds). This step was repeated before two successive filtrations (70 µm and 40 µm). Digestion was stopped by adding 45 mL of cold PBS to the cells. After centrifugation (300 x g for 5 minutes at 4°C), the cells were resuspended in 5 mL FACS buffer prepared in PBS supplemented with 3% fetal bovine serum (ThermoFisher Scientific, cat#10270-106), 2 mM EDTA, and 10 µM Y27632. Cell concentration was measured using an automated cell counter Countess 3 (ThermoFisher Scientific, cat#AMQAX2000).

Cell preparation for single-cell sequencing

Cells (2.10^6) were centrifuged (300 x *g* for 5 minutes at 4°C) and resuspended in 1mL of PBS supplemented with 10 µM Y27632 in PBS, twice. Dead cells were stained with the LIVE/DEAD™ Fixable Violet Dead Cell Stain Kit (ThermoFisher Scientific, cat#L34963), according to the manufacturer instructions. After 30 minutes of incubation (4°C, protected from light), cells were centrifuged (300 x *g* for 5 minutes at 4°C) and resuspended in 1 mL FACS buffer, twice. Cells were filtered (40 µM) and sorted (10^5 live and single) in a 1.5 mL tube containing 10 µL of PBS supplemented with 10 µM Y27632 by using a BD Influx cell sorter instrument with a 100µm nozzle, under 20psi at the I2MC Cytometry and Cell sorting TRI platform (Toulouse, France). After centrifugation (300 x *g* for 5 minutes at 4°C), cells were resuspended in 100 µL PBS, counted manually and their viability was verified by trypan blue staining.

Single-cell sequencing

For single cell RNA-sequencing, approximately 10,000 cells per sample were encapsulated into droplets using Chromium Next GEM Single Cell 3′ Reagent Kits v3.1 according to manufacturer's protocol (10× Genomics CG000315 Rev E user guide). Briefly, after generation of Gel bead-in-EMulsions (GEMs) using Next GEM Chip G, GEMs were reverse transcribed in a C1000 Touch Thermal Cycler (BioRad) programed at 53°C for 45 min, 85°C for 5 min, and held at 4°C. After reverse transcription, single-cell droplets were broken and cDNA was isolated and cleaned with Cleanup Mix containing DynaBeads (Thermo Fisher Scientific). cDNA was then amplified with a C1000 Touch Thermal Cycler programed at 98°C for 3 min, 12 cycles of (98°C for 15 s, 63°C for 20 s, 72°C for 1 min), 72°C for 1 min, and held at 4°C. Subsequently, approximately 250 ng of amplified cDNA was fragmented, end-repaired, A-tailed, index adaptor ligated, and cleaned with cleanup mix containing SPRIselect Reagent Kit (Beckman Coulter) in between steps. Post-ligation product was amplified and indexed with a C1000 Touch Thermal Cycler programed at 98°C for 45 s, 11 cycles of (98°C for 20 s, 54°C for 30 s, 72°C for 20 s), 72°C for 1 min, and held at 4°C. The sequencing-ready libraries were cleaned up with SPRIselect beads. 10x libraries were pooled and charged with 1% PhiX on one S1 lane of the NovaSeq 6000 instrument (Illumina) using the NovaSeq 6000 S1 Reagent Kit v1.5 (100 cycles), and the following sequencing parameters: 28 bp read 1 – 10 bp index 1 (i7) – 10 bp index 1 (i5) – 150 bp read 2. The S1 lane generated a total of 810.10^6 raw reads.

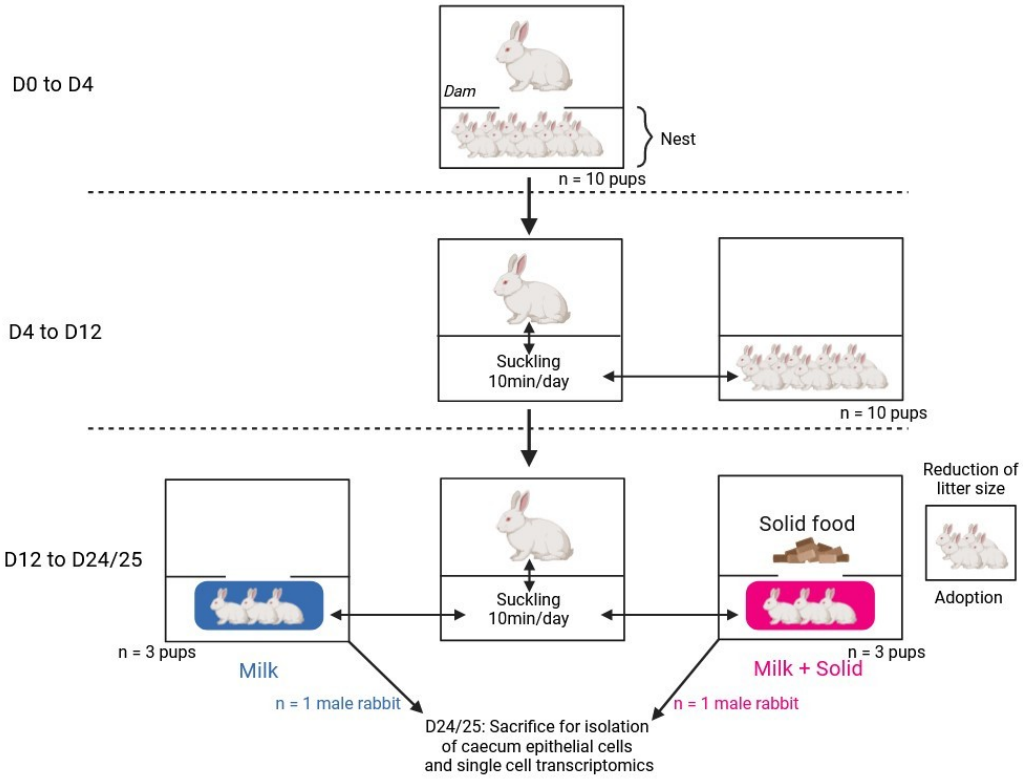

Figure 1: Schematic representation of the experimental design represented for one litter (n=4 litters in total).