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Genomics, Proteomics, and Metabolomics

## Specific Milk Composition of *miR-30b* Transgenic Mice Associated with Early Duodenum Maturation in Offspring with Lasting Consequences for Growth

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### ABSTRACT

**Background:** Milk composition is complex and includes numerous components essential for offspring growth and development. In addition to the high abundance of *miR-30b* microRNA, milk produced by the transgenic mouse model of *miR-30b*-mammary deregulation displays a significantly altered fatty acid profile. Moreover, wild-type adopted pups fed *miR-30b* milk present an early growth defect.

**Objective:** This study aimed to investigate the consequences of *miR-30b* milk feeding on the duodenal development of wild-type neonates, a prime target of suckled milk, along with comprehensive milk phenotyping.

**Methods:** The duodenums of wild-type pups fed *miR-30b* milk were extensively characterized at postnatal day (PND)-5, PND-6, and PND-15 using histological, transcriptomic, proteomic, and duodenal permeability analyses and compared with those of pups fed wild-type milk. Milk of *miR-30b* foster dams collected at mid-lactation was extensively analyzed using proteomic, metabolomic, and lipidomic approaches and hormonal immunoassays.

**Results:** At PND-5, wild-type pups fed *miR-30b* milk showed maturation of their duodenum with 1.5-fold ( $P < 0.05$ ) and 1.3-fold ( $P < 0.10$ ) increased expression of *Claudin-3* and *Claudin-4*, respectively, and changes in 8 duodenal proteins ( $P < 0.10$ ), with an earlier reduction in paracellular and transcellular permeability (183 ng/mL fluorescein sulfonic acid [FSA] and 12 ng/mL horseradish peroxidase [HRP], respectively, compared with 5700 ng/mL FSA and 90 ng/mL HRP in wild-type;  $P < 0.001$ ). Compared with wild-type milk, *miR-30b* milk displayed an increase in total lipid (219 g/L compared with 151 g/L;  $P < 0.05$ ), ceramide (17.6  $\mu$ M compared with 6.9  $\mu$ M;  $P < 0.05$ ), and sphingomyelin concentrations (163.7  $\mu$ M compared with 76.3  $\mu$ M;  $P < 0.05$ ); overexpression of 9 proteins involved in the gut barrier ( $P < 0.1$ ); and higher insulin and leptin concentrations (1.88 ng/mL and 2.04 ng/mL, respectively, compared with 0.79 ng/mL and 1.06 ng/mL;  $P < 0.01$ ).

**Conclusions:** *miR-30b* milk displays significant changes in bioactive components associated with neonatal duodenal integrity and maturation, which could be involved in the earlier intestinal closure phenotype of the wild-type pups associated with a lower growth rate.

**Keywords:** milk composition, intestinal barrier, early maturation, mice, *miR-30b*

**Abbreviations:** CAN, acetonitrile; ApoA1, apolipoprotein A-I; ApoC1, apolipoprotein C-I; Asah2, neutral ceramidase; Catz, cathepsin Z; Cdh1, E-cadherin; Cer, ceramide; CID, collision induced dissociation; Ctna1, alpha-1 catenin; FA, formic acid; FAA, free amino acid; Flna, filamin A; FSA, fluorescein sulfonic acid; Glcm1, glycosylation-dependent cell adhesion molecule 1; H4, histone H4; HRP, horseradish peroxidase; IPA, ingenuity pathway analysis; IS, internal standard; LD, lactation day; Lmna, isoform C of prelamin-A/C; Lrp1, prolown-density lipoprotein receptor-related protein 1; MRM, multiple reaction monitoring; Mup2, major urinary protein 2; NelfB, negative elongation factor B; Ospn, osteopontin; PCA, principal component analysis; PND, postnatal day; Rbp2, retinol-binding protein 2; Saa, serum amyloid protein A; S1P, sphingosine-1-phosphate; SM, sphingomyelin; SP-D, pulmonary surfactant-associated protein D; Tena, tenascin; TFA, trifluoroacetic acid; Tjp1, tight junction protein 1; WT, wild-type; ZO-1, zonula occludens 1.

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## Introduction

The complex and dynamic composition of breast milk includes bioactive components that are essential for offspring growth and development [1–3]. The milk concentrations of macronutrients such as lipids, proteins, and carbohydrates have been heavily studied [4–6] and shown to correlate with infant weight gain and body mass index [7]. In addition, milk provides a wide variety of other components, and their biological activity and impact on infant growth are gradually being investigated. In particular, free amino acids (FAAs), such as arginine and tyrosine, have been identified as robust milk biomarkers with good predictive capacity for weight gain during the first weeks of life in preterm children [8]. Additionally, studies have shown that milk leptin and insulin are involved in the regulation of early growth of pups [2,9–12].

In connection with pup growth modulation and overall development, milk components play an important local role in gastrointestinal neonatal development [13,14]. Lipids and fatty acids have been shown to influence this process [14–16], and the provision of essential nutrients is beneficial due to their contribution to the structural and functional maturation of the pup intestine [13,17–21]. Notably, *in vivo* studies on rodents have shown that long-chain polyunsaturated fatty acid exposure and intestinal levels impact postnatal gut maturation [20], particularly through the modulation of the levels of proteins involved in intestinal tight junctions (such as Claudin-3 or tight junction protein 1 [Tjp1]), cell differentiation, and lipid metabolism [19,22,23]. In addition, among other bioactive compounds, the intake of milk insulin and leptin was shown to strengthen gut barrier development and reduce intestinal permeability [14,24,25].

The tight junctions formed between the apical areas of intestinal epithelial cells are critical for the maintenance of gut permeability and intestinal barrier function through the regulation of the paracellular movement of molecules between the intestinal lumen and subepithelial tissues [26–28]. The transmembrane proteins claudins and occludin and the cytoplasmic protein zonula occludens 1 (ZO-1) mediate this movement [26, 29–31]. In rodents, intestinal permeability is high during the fetal and neonatal periods and is drastically reduced due to the decreased endocytic capacity of small intestine cells at 2 wk of life [32,33]. By 3 wk of life, ie, at weaning, the intestinal maturation process is finished, and gut closure is completed.

We previously reported that, in addition to a high abundance of the *miR-30b* microRNA, the milk produced by the transgenic mouse model of *miR-30b* mammary deregulation [34] displays a unique fatty acid profile, with increased MUFA levels and decreased SFA levels [35,36]. This alteration was associated with a significant growth defect in wild-type (WT) pups suckled by *miR-30b* dams, which is noticeable from a very early age (3 postnatal days [PNDs]) [34]. For this reason, *miR-30b* milk is a good model to study the impact of milk compositional changes on the intestinal maturation of pups. Therefore, using a cross-fostering rodent model, we relied on *miR-30b* transgenic mice to investigate the early consequences of suckling *miR-30b* milk on neonatal duodenal development and further characterize its specific milk composition compared with WT milk. The long-term impact of *miR-30b* milk nursing was also evaluated through growth monitoring of WT offspring fed with *miR-30b* or WT milk until adulthood.

## Materials and Methods

### Experimental animals

All animal manipulations were performed in strict accordance with the guidelines of the Code for Methods and Welfare Considerations in Behavioral Research with Animals (Directive 86/609EC), and the recommendations of the French Commission de Génie Génétique (Permit number 12931 (01.06.2003)), who approved this study. Animals were bred in a controlled environment at 20°C to 23°C, 45% to 55% humidity, and 12-h light:dark cycle in a conventional animal facility, and every effort was made to minimize animal suffering. During the experiment, mice were carefully monitored, in particular according to the humane endpoints score sheets [37], and no animal needed to be excluded. Mice were fed *ad libitum* with water and a commercial complete diet developed for mouse breeding, containing 22% proteins, 4.5% fat, 3.9% fiber, 6.7% crude ash, minerals, and vitamins (V1124-000 ssniff M-Z, Complete feed for mice - breeding, ssniff Spezialdiäten GmbH). Transgenic *miR-30b* mice (FVB/N strain background) were previously described [34]. Two experimental groups were compared in this study: WT females nursing WT offspring (control group) and *miR-30b* transgenic females nursing WT offspring (*miR-30b* group). At 6 to 8 wk of age, WT and *miR-30b* females were mated with WT FVB/N males. At birth, offspring from WT females were mixed and randomly assigned to WT and *miR-30b* dams. Foster litters were limited to 8 pups *per* female, to optimize lactation, while ensuring a balanced sex ratio in each litter. Each female was bred with its adopted litter in an individual cage. All females successfully fed their litters, and none was excluded from the experiment due to lactation failure. Growth curve analysis aside (described below), a total of 15 WT and 17 *miR-30b* dams and 48 WT pups were analyzed in the present study. They were part of a complete experimental protocol involving 41 WT and 21 *miR-30b* dams, mated with 10 WT males, and a total of 328 WT pups were fed (Supplemental Table 1). The day of parturition was counted as day 0 of lactation. Days are termed LD (lactation day) or PND, depending on whether the day refers to the stage of lactation or life of the pup. The list of specific sections of the manuscript containing information relating to items of the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines 2.0 [38] is provided in Supplemental Table 2.

### Growth curves and feed intake measurements

To evaluate the impact of nursing with *miR-30b* milk on the long-term growth of mice, a total of 46 WT pups were used and evenly divided for adoption at birth by 3 WT and 3 transgenic *miR-30b* mothers. Male and female pups were distributed equally under each nursing dam, with 3 to 5 males and females under each mother and a limit of 8 pups per litter. Mice were weighed individually 2 times per week until PND-153. In addition, individual feed intake was recorded for the adult pups from PND-118 to PND-153, measured as the difference in weight (g) between the food pellets served and those remaining at the next weighing.

### Intestinal characterization

#### Tissue collection

At PND-5, PND-6, and PND-15, WT pups fed either WT or *miR-30b* milk were euthanized via cervical dislocation, and the number of male and female pups was balanced. The duodenal

part of the small intestine was excised, and the tract was flushed with ice-cold sterile physiological solution. For transcriptomic and proteomic analysis, samples were flash frozen in dry ice or liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until isolation. For histological analysis, samples were fixed in 4% paraformaldehyde for 10 min at  $4^{\circ}\text{C}$ , transferred to ethanol solutions (50% for 1 h, room temperature, and then 70% at  $4^{\circ}\text{C}$ ), and embedded in paraffin. For permeability analysis, fresh collected samples were immediately placed in 500  $\mu\text{L}$  of physiological serum, kept on ice, and then transferred to an Ussing chamber.

### Histological analysis

Five-micrometer sections of paraffin-embedded duodenum samples from 5 pups fed WT milk and 5 with *miR-30b* milk at PND-5 and PND-15 were mounted on slides. The slides were stained with hematoxylin and eosin and Alcian Blue-periodic acid-Schiff for the analysis of acid and neutral mucins [39] and then digitized under bright light using a Hamamatsu NanoZoomer (Hamamatsu Photonics) by the INRAE @BRIDGE platform.

### Transcriptomic analysis

Total RNA was isolated with the RNA Now kit (Ozyme) with an overnight isopropanol precipitation from duodenum biopsies of 5 pups fed WT milk and 5 with *miR-30b* milk at PND-5 and PND-15. The concentration and integrity of the RNA were assessed by spectrophotometry (Nanodrop, ND-1000) and using the Total RNA Nano kit on a BioAnalyser 2100 (Agilent Technologies). The RNA samples were stored at  $-80^{\circ}\text{C}$  until further processing.

Total RNA (2.5  $\mu\text{g}$ ) was reverse transcribed using SuperScript IV Vilo Master Mix (Fisher Scientific) supplemented with 10  $\mu\text{L}$  DNase and RNase-free water in a reaction mixture containing 4  $\mu\text{L}$  Enzyme Mix and 6  $\mu\text{L}$  DNase and RNase-free water and incubated at  $25^{\circ}\text{C}$  for 10 min,  $50^{\circ}\text{C}$  for 10 min, and  $85^{\circ}\text{C}$  for 5 min. cDNA was stored at  $-20^{\circ}\text{C}$  until further use.

Quantitative PCR assays were performed on a QuantStudio 12K Flex (Thermo Fisher) in a reaction containing 10  $\mu\text{L}$  of Master Mix PCR Power SYBR Green (Thermo Fisher), 1.2  $\mu\text{L}$  of 5  $\mu\text{M}$  forward primer, 1.2  $\mu\text{L}$  of 5  $\mu\text{M}$  reverse primer, and 5  $\mu\text{L}$  of cDNA diluted 1:10 in a total volume of 20  $\mu\text{L}$ . Quantitative PCR cycling conditions were  $50^{\circ}\text{C}$  for 2 min,  $95^{\circ}\text{C}$  for 10 min, 40 cycles of  $95^{\circ}\text{C}$  for 15 sec, and  $60^{\circ}\text{C}$  for 1 min. Melting curves were obtained using a step at  $95^{\circ}\text{C}$  for 15 sec,  $60^{\circ}\text{C}$  for 15 sec, and a step where the temperature was increased to  $95^{\circ}\text{C}$  at a rate of  $0.05^{\circ}\text{C}/\text{s}$ . Primer efficiency was tested on 3 cDNA dilutions (1:10, 1:100, 1:1000) and on a no-template control. Efficiency was calculated with the slope of the standard curve as follows:  $E = 10^{(-1/\text{slope})} - 1$ . Primers were validated with an efficiency of 80% to 110%. After normalization with *GAPDH* to calculate the relative expression of the genes, the data were analyzed using the Delta-Delta Ct method ( $2^{-\Delta\Delta\text{Ct}}$ ) [40]. The primer sequences used for the amplification of *Claudin-1*, *Claudin-3*, *Claudin-4*, and *GAPDH* are presented in Supplemental Table 3.

### Proteomic analysis

Frozen duodenal biopsies from 5 pups fed WT milk and 5 pups fed *miR-30b* milk at PND-5 and PND-15 were homogenized in 1 mL of cold lysis buffer (10 mM Tris pH 7.2 supplemented with cComplete EDTA-free protease inhibitors [Roche]) and centrifuged

at  $2300 \times g$  and  $4^{\circ}\text{C}$  for 10 min. The proteins contained in the supernatant were precipitated with a mixture of 10% trichloroacetic acid and 0.07%  $\beta$ 2-mercaptoethanol in acetone (1.8 mL of precipitation solution for 200  $\mu\text{L}$  of sample) and incubated overnight at  $-20^{\circ}\text{C}$  followed by centrifugation at maximum speed ( $16,100 \times g$ ) at  $4^{\circ}\text{C}$  for 20 min. After removal of the supernatant, the precipitate was rinsed with 1.8 mL of a solution of 0.07%  $\beta$ 2-mercaptoethanol in acetone stored at  $-20^{\circ}\text{C}$ , which was vortexed 4 times within 1 h before being incubated at  $-20^{\circ}\text{C}$  overnight. After centrifugation at  $9300 \times g$  for 15 min at  $4^{\circ}\text{C}$ , the supernatant was removed, and the precipitate was left to dry at room temperature for 5 min. Finally, the proteins were resuspended in 30  $\mu\text{L}$  of solubilization buffer (6 M urea, 1 M thiourea, 0.1% RapiGest SF Surfactant [Waters],  $1 \times$  cComplete EDTA-free protease inhibitors [Roche] in 10 mM Tris-HCl pH 7.6). Protein concentrations were measured according the Quick Start Bradford Protein Assay (Bio-Rad), and the samples were stored at  $-80^{\circ}\text{C}$ .

To perform a shotgun LC-MS/MS total proteome analysis of pup duodenal tissue, a double enzymatic digestion was performed on all samples. Potential disulfide bridges were reduced with 10 mM dithiothreitol, and free cysteines were alkylated with 50 mM iodoacetamide. Then, 30  $\mu\text{L}$  of 25 mM  $\text{NH}_4\text{HCO}_3$  was added to dilute the urea to 3 M. A first digestion was performed with LysC with an enzyme/substrate ratio of 1:50 w/w (Wako Chemicals) at  $37^{\circ}\text{C}$  for 3 h. The sample was diluted to 1 M urea, and the second digestion was performed with trypsin at an enzyme/substrate ratio of 1:50 w/w (Promega, V5111) overnight at  $37^{\circ}\text{C}$ . To quench digestion, the pH was adjusted to 2 by adding 2  $\mu\text{L}$  of 30% trifluoroacetic acid (TFA) (v/v).

Then, total peptides were purified on C18 SPE cartridges (Strata X 30 mg/1 mL [Phenomenex]) previously activated with acetonitrile (ACN) and equilibrated with 0.1% TFA. The samples were washed with 0.1% TFA and eluted twice with 500  $\mu\text{L}$  of 40% ACN/0.1% TFA. The purified peptide extracts were dried in a concentrator and then reconstituted in different volumes of chromatographic loading buffer (2% ACN + 0.08% formic acid [FA]) to obtain a solution at 0.2  $\mu\text{g}/\mu\text{L}$  for MS analysis.

HPLC was performed on a Nano-HPLC (Eksigent). Buffers A and B were prepared with 0.1% FA in water and 0.1% FA in ACN, respectively. A 4  $\mu\text{L}$  sample of the peptide solution was loaded at 7.5  $\mu\text{L}/\text{min}$  for 1 min on a Biosphere C18 trap column (particle size: 5  $\mu\text{m}$ , pore size: 12 nm, inner/outer diameters: 360/100  $\mu\text{m}$ , length: 20 mm; NanoSeparations) and desalted with 0.1% FA and 2% ACN in water. Then, peptides were separated on a Biosphere C18 column (particle size: 3  $\mu\text{m}$ , pore size: 12 nm, inner/outer diameters: 360/75  $\mu\text{m}$ , length: 300 mm; NanoSeparations). Peptide separation was achieved at 300 nL/min with a linear gradient from 5% to 35% buffer B for 180 min.

Eluted peptides were analyzed online with a Q-Exactive mass spectrometer (Thermo Fisher Scientific) using a nano-electrospray interface. Ionization (1.5 kV ionization potential) was performed with a glass needle (noncoated capillary silica tips, 360/20-10; New Objective). Peptide ions were analyzed using Xcalibur 2.07 with the following data-dependent acquisition steps: 1) full MS scan (mass-to-charge ratio [m/z], 400 to 1400; profile mode) with a resolution of 70,000 and 2) MS/MS (isolation window = 3 m/z, automatic gain control (AGC) target =  $5e4$ , max ion time = 120 ms, collision energy = 27%; profile mode, resolution = 17,500). Step 2 was repeated for the 8 major

ions detected in step 1. Dynamic exclusion was set to 40 s. Only the doubly and triply charged precursor ions were subjected to MS/MS fragmentation.

Xcalibur raw data were transformed to mzXML open source format and centroided using the msconvert software in the ProteoWizard 3.0.4601 package [41]. Protein identification was performed using the X! Tandem Piledriver (version 2015.04.01.1; [www.thegpm.org](http://www.thegpm.org)) using the *Mus musculus* UniProt/SwissProt database [42] (v.20105.10.14; 25,248 entries). Proteins identified were filtered and grouped using X! Tandem Pipeline [43] (v3.3.4 and v.3.3.5/2) ([http://pappso.inrae.fr/bioinfo/xtandempipeline/news/2019\\_xtpcpp\\_0236](http://pappso.inrae.fr/bioinfo/xtandempipeline/news/2019_xtpcpp_0236)).

Data filtering was achieved according to a peptide E-value < 0.01, protein log (E-value) less than -4 and a minimum of 2 identified peptides per protein. MS peaks were detected and aligned using MassChroQ and MassChroQR [44]. The relative quantification of protein abundances was performed using the method of spectral counting defined as the number of MS spectra assigned to a protein [45]. The MS proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository [46] with the dataset identifier PXD037626.

### Duodenal permeability assays

Because tight junctions play an important role in trans-epithelial transport, the intestinal permeability of 6-d-old pups nursed with WT ( $n = 5$ ) or *miR-30b* milk ( $n = 5$ ) was analyzed by mounting duodenum pieces (0.5 cm<sup>2</sup>) in Ussing chambers [47]. The barrier function was evaluated by measuring paracellular and transcellular permeability to 4 kDa FSA-FITC (fluorescein sulfonic acid-fluorescein isothiocyanate; Fisher Scientific 11564756) and 44 kDa HRP type II (horseradish peroxidase; Sigma P8250), respectively. At sampling time  $t=0$ , 2 mL of warm 10 mM Krebs glucose was added to the Ussing chamber on the serous side of the insert (Warner Instruments P2403), and 1.94 mL of warm 10 mM Krebs mannitol was added on the mucosal side along with 50  $\mu$ L of 10 mg/mL HRP and 10  $\mu$ L 10 mg/mL FSA. Oxygenation and a temperature of 37°C were constantly maintained. Two 200- $\mu$ L samples were collected on the serosal side every 15 min for 1.5 h, and the volume of 10 mM Krebs glucose was readjusted to 2 mL at each collection. At each time point, 200  $\mu$ L (100  $\mu$ L in duplicate) were placed in a black 96-well plate (Greiner Bio One #655076) for FSA quantification, and 200  $\mu$ L (100  $\mu$ L in duplicate) were added to a transparent 96-well plate (Greiner BioOne #655101) for HRP assay. FSA fluorescence measurement was performed on a Tecan instrument (Infinite M200 Pro, Tecan) with Infinite 200Pro software at an excitation wavelength of 488 nm and emission of 520 nm. The HRP assay was performed after reaction with 160  $\mu$ L/well of a solution consisting of 1.8 mg/mL Na<sub>2</sub>HPO<sub>4</sub> (Merck S9763), 13.7 mg/mL NaH<sub>2</sub>PO<sub>4</sub> (Merck S0751), 0.08 mg/mL o-dianisidine dihydrochloride (Sigma D3252), and 0.003% H<sub>2</sub>O<sub>2</sub> (Sigma H1009), incubated for 10 min, and then stopped by the addition of 100  $\mu$ L of 0.1% sodium azide per well. Absorbance measurements were then performed on a Tecan instrument at 405 nm.

### Milk composition

#### Milk sampling

For the collection of milk samples, the pups were removed from their mothers (4 h beforehand) to obtain sufficient milk.

Dams received an intraperitoneal injection of 150  $\mu$ L oxytocin (10 IU/mL, Ocytovem, Med'Vet) to stimulate milk ejection, and were anesthetized after 10 min using a 0.12% xylazine (Rompun, Bayer)/10 mg/mL ketamine (Imalgène, Merial) mix. Milking was facilitated by applying manual pressure. All samples were collected at mid-lactation (LD-15) and then frozen for each individual as aliquots of 20 to 100  $\mu$ L of whole milk and aliquots of 150 to 500  $\mu$ L of milk diluted at a ratio of one volume of H<sub>2</sub>O for 3 volumes of milk. Milk was skimmed by centrifugation at 1500  $\times$  g at 4°C for 15 min. Whole and skimmed milk samples were then stored at -80°C until use.

### Protein and proteomic analysis

The total protein content of skimmed milk was measured using the Quick Bradford kit (Bio-Rad #5000201) according to the manufacturer's recommendations from aliquots of 20  $\mu$ L of milk diluted 1:10 for 4 individual samples per group at LD-15.

Each sample was diluted to 1  $\mu$ g/ $\mu$ L in a final volume of 50  $\mu$ L of a buffer composed of 6 M urea, 1 M thiourea, and 1  $\times$  cOmplete EDTA-free protease inhibitors (Roche) in 10 mM Tris-HCl pH 7.2. Samples in solution were subjected to an initial disulfide bridge reduction step using 10 mM dithiothreitol at 56°C for 30 min. Then, alkylation of free cysteines was performed with 50 mM iodoacetamide at room temperature for 1 h in the dark. Digestion was performed with 0.5  $\mu$ g lysC for 3 h followed by 0.5  $\mu$ g trypsin overnight at 37°C, and the reaction was stopped by adding 0.1% TFA. Then, the peptides were purified on C18 SPE cartridges (Strata X 30 mg/1 mL [Phenomenex]) and eluted with 500  $\mu$ L of 40% I/0.1% TFA. Peptide extracts were dried with a speed-vac and then reconstituted in 80  $\mu$ L of chromatographic loading buffer (2% ACN + 0.1% FA). Aliquots of each sample were added to different volumes of chromatographic loading buffer (2% ACN + 0.08% FA) to obtain a solution at 0.1  $\mu$ g/ $\mu$ L for mass spectrometry analysis.

A 4- $\mu$ L sample was loaded at 20  $\mu$ L/min on a precolumn ( $\mu$ -Precolumn, 300  $\mu$ m inner diameter  $\times$  5 mm, C18 PepMap100, 5  $\mu$ m, 100 Å; Thermo Fisher) and washed with loading buffer. After 3 min, the precolumn cartridge was connected to the separating column (Acclaim PepMap, 75  $\mu$ m  $\times$  500 mm, C18, 3  $\mu$ m, 100 Å; Thermo Fisher). Buffer A consisted of 0.1% FA in 2% ACN, and buffer B consisted of 0.1% FA in 80% ACN. Peptide separation was achieved at 300 nL/min with a linear gradient from 1% to 45% buffer B for 120 min.

Peptide ions were analyzed using Xcalibur 3.1.66.10 with the following machine setup in CID (collision induced dissociation) mode: 1) full MS scan in Orbitrap (scan range [m/z] = 400–1500) and 2) MS/MS using CID (35% collision energy) in Ion Trap (AGC target =  $4.0 \times 10^3$ , maximum injection time = 300 ms, data type = centroid). The analyzed charge states were set to 2 to 4, the dynamic exclusion was set to 60 s, and the intensity threshold was fixed at  $5.0 \times 10^3$ .

Protein identification was performed using X! Tandem Piledriver (version 2015.04.01.1), X! Tandem Pipeline (version 3.4.2), and the *Mus musculus* UniProt/SwissProt database (version January 2017; 35,208 entries). Data filtering was achieved according to a peptide E-value < 0.01, protein log (E-value) less than -4 and a minimum of 2 identified peptides per protein. MS peaks were detected and aligned using MassChroQ and MassChroQR. The relative quantification of protein abundance was performed using the method of spectral counting defined as the number of MS spectra assigned to a protein. The

mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD037629.

### Metabolomic and lipidomic analysis

Targeted and quantitative analyses of FAAs were performed on 9 individual samples of WT milk and 7 samples of *miR-30b* milk using a slightly modified LC–MS/MS method [48]. Briefly, individual stock solutions (10 to 20 mM) of unlabeled amino acid standards were prepared in 0.1 M HCl, and a pool of unlabeled standard solutions was prepared and serially diluted in HCl (histidine, isoleucine, leucine, lysine, methionine, threonine, tryptophan, valine, arginine, alanine, aspartate, asparagine, glutamine, glutamate, proline, glycine, serine, tyrosine) (Sigma–Aldrich) to obtain a standard solution with the following range: 0.006 to 100  $\mu\text{mol/L}$ . A solution of labeled internal standard (IS) solution consisting of 300  $\mu\text{mol/L}$  (IS:  $^{15}\text{N}$ -aspartic acid,  $^{15}\text{N}$ -glutamic acid,  $^{13}\text{C}$ -alanine,  $^{13}\text{C}$ -arginine,  $^{15}\text{N}_2$ -asparagine,  $\text{d}_3$ -cysteine,  $^{15}\text{N}$ -glutamine,  $^{13}\text{C}$ -glycine,  $^{15}\text{N}_3$ -histidine,  $^{15}\text{N}$ -leucine,  $^{13}\text{C}_6$ -lysine,  $^{13}\text{C}$ - $\text{d}_3$ -methionine,  $\text{d}_5$ -phenylalanine,  $^{13}\text{C}$ -proline,  $^{15}\text{N}$ -serine,  $^{15}\text{N}$ -threonine,  $\text{d}_5$ -tryptophan,  $^{15}\text{N}$ -tyrosine,  $^{13}\text{C}$ -caline) (Cambridge Isotope Laboratories) was prepared in water. The standard solution and milk samples (20  $\mu\text{L}$ ) were then extracted with 100  $\mu\text{L}$  of methanol and 15  $\mu\text{L}$  of IS solution. The samples were mixed and centrifuged at  $10,000 \times g$  at  $10^\circ\text{C}$  for 15 min to remove the precipitated proteins. The supernatants were collected, dried, and derivatized with 100  $\mu\text{L}$  of a butanol solution containing 5% acetyl chloride and kept at  $60^\circ\text{C}$  for 30 min. The solvent was then removed under a gentle stream of nitrogen ( $60^\circ\text{C}$ ). The dried samples were dissolved in 100  $\mu\text{L}$  of water containing 0.1% FA, and 10  $\mu\text{L}$  was injected into a reversed-phase column (Acquity BEH-C18, 1.7  $\mu\text{m}$ ;  $2.1 \times 100$  mm, Acquity H-Class UPLCTM device, Waters Corporation) held at  $60^\circ\text{C}$ . The compounds were separated with a linear gradient of mobile phase B (0.1% FA in methanol) in mobile phase A (0.1% FA in water) at a flow rate of 400  $\mu\text{L}/\text{min}$ . The target compounds were then detected by a Xevo Triple-Quadrupole mass spectrometer with an electrospray ionization interface operating in the positive ion mode with multiple reaction monitoring (MRM).

Targeted and quantitative analyses of milk sphingolipids were performed on 4 individual samples of WT milk and 4 samples of *miR-30b* milk using reversed-phase LC–MS/MS (Xevo TQD mass spectrometer coupled to an ACQUITY H-Class UPLC System, Waters Corporation) with MRM mode as previously described [49]. Briefly, a pool of reference standard solutions, including sphingosine-1-phosphate (S1P;  $\text{d}18:1$ ), 9 ceramide (Cer) species, and 9 sphingomyelin (SM) species (Avanti Polar Lipids), was prepared with serial dilutions of methanol to obtain 7 standard solutions ranging from 1 to 500 nmol/L, 2 to 1000 nmol/L, and 0.04 to 20 mmol/L for Cer, S1P, and SM, respectively. Standard solutions and milk samples (10  $\mu\text{L}$ ) were then extracted with 500 mL of methanol/chloroform mixture (2:1, v/v) containing exogenous ISs [Cer ( $\text{d}18:1/17:0$ ) 500 nmol/L; S1P ( $\text{d}17:1$ ) 500 nmol/L; and SM ( $\text{d}18:1/17:0$ ) 5 mmol/L]. Cer and SM profiles were analyzed using reversed-phase LC–MS/MS (Xevo TQD mass spectrometer coupled to an ACQUITY H-Class UPLC System, Waters Corporation) in MRM mode. Data acquisition and analyses were performed with MassLynx1 and TargetLynx1 version 4.1 software, respectively (Waters Corporation). Recoveries were assessed with ISs and were  $>91\%$ .

### Lactose analysis

The lactose content of skim milk was measured using the K6LACGAR Assay kit (Megazyme) according to the manufacturer's recommendations for 4 samples of WT milk and 8 samples of *miR-30b* milk diluted 1:50, each assayed in triplicate.

### Hormone analysis

Leptin quantification of skimmed milk ( $n = 4$  WT and  $n = 5$  *miR-30b*) and total milk ( $n = 4$  WT and  $n = 4$  *miR-30b*) was performed using a Mouse Leptin ELISA Kit (Crystal Chem #90030). Insulin quantification of total milk ( $n = 5$  WT and  $n = 5$  *miR-30b*) was performed using the Low Range Assay (0.1–6.4 ng/mL) of the Mouse Insulin ELISA Kit (Crystal Chem #90080). For every analysis, 5  $\mu\text{L}$  of each sample, diluted at a ratio of one volume of  $\text{H}_2\text{O}$  for 2 volumes of milk, was assayed in duplicate according to the manufacturer's instructions. Absorbance measurements (measure A450 nm and subtract A630 nm) were performed on a TriStar<sup>2</sup> S LB 942 multimode microplate reader (Berthold Technologies).

### Statistical analysis

For duodenal transcriptomics analysis and milk metabolic, lactose, and hormone analysis, data are presented as the mean  $\pm$  SEM, and significant differences between the WT and *miR-30b* groups were assessed using nonparametric Mann–Whitney tests. Test results were considered statistically significant when  $P$  values were less than 0.05.

For duodenal and milk proteomics analysis, a principal component analysis (PCA) of the data was performed using R software v3.2.4 (R Development Core Team, 2018, <http://www.R-project.org>) with the ade4 package [50]. Differential expression analysis between proteomic spectra counts was performed according to the WT or *miR-30b* milk with the Limma package [51] after normalization by a voom transformation of the data (mean-variance modeling at the observational level). In Limma, rowwise linear models were applied using an empirical Bayes shrinkage procedure. The  $P$  values were adjusted for multiple testing using the Benjamini and Hochberg method [52], and those with an adjusted  $P$  value less than 0.05 were considered significant.

Growth curve data statistical analyses were performed using R software v3.2.5 with a linear mixed-effects model, considering maternal milk, time, sex, sex:time, and milk:time as fixed effects and animals as random effects and with an autoregressive correlation structure of order 1.  $P$  values less than 0.05 were considered significant.

## Results

### Pups fed *miR-30b* milk show accelerated maturation of their duodenum with earlier reduced paracellular and transcellular permeability

Because milk components can have an impact on the intestinal development of offspring, a broad characterization of the duodenum of WT pups fed *miR-30b* milk was performed at PND-5 and PND-15 and compared to pups fed WT milk. Duodenal sections of the pups nursed by transgenic *miR-30b* foster dams did not reveal any significant structural changes either at PND-5 or PND-15, and they also showed no differences in mucus cell counts at PND-15 (Figure 1). At PND-5, the duodenum of pups

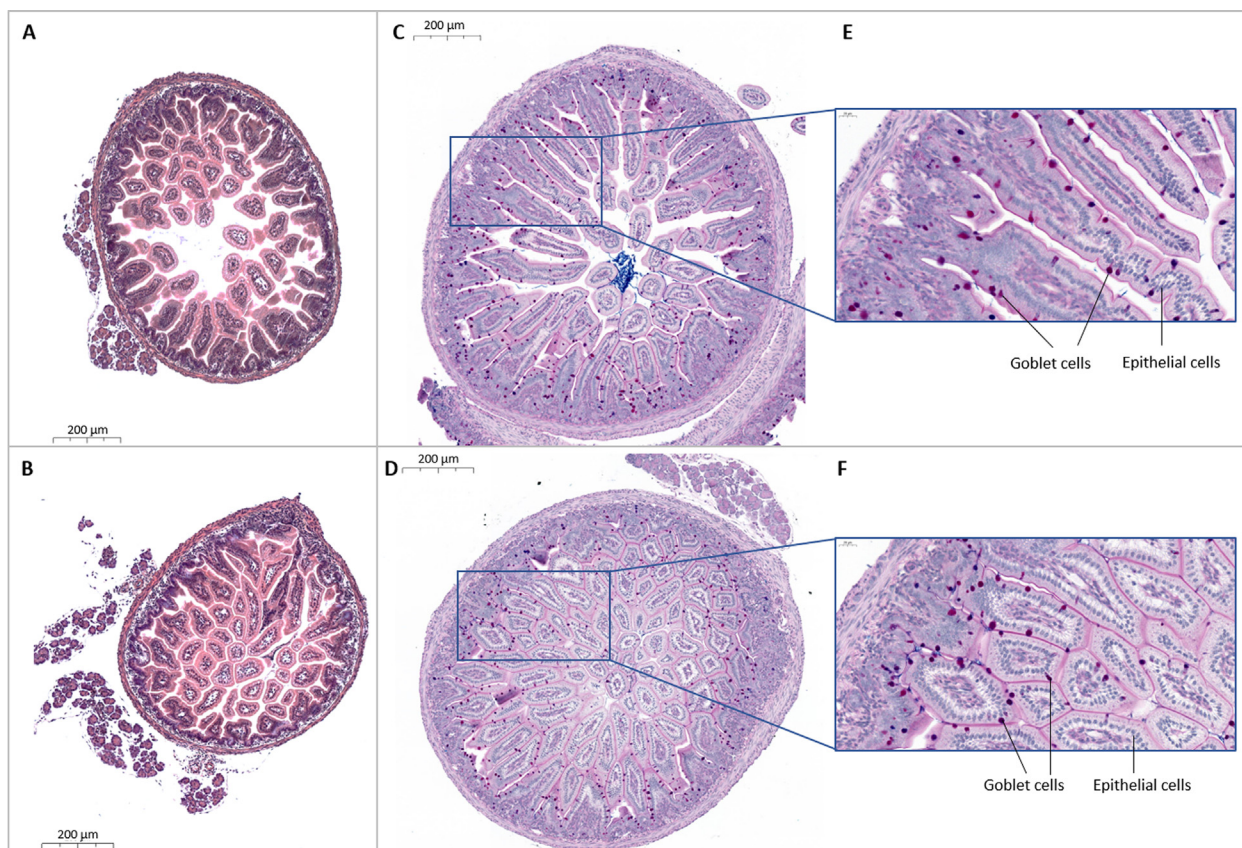
fed *miR-30b* milk showed a significant increase in the expression of the proteins *Claudin-3* (1.5-fold [ $P < 0.05$ ]) and 1.3-fold trend for *Claudin-4* [ $P < 0.10$ ], which are involved in forming tight junctions between epithelial cells, compared with pups fed WT milk (Figure 2). Considering the longitudinal analysis of protein expression, *Claudin-1* expression in WT milk-fed pups was strongly reduced (ratio of *Claudin-1/GAPDH* of 0.74) at PND-15 compared to its level at PND-5, whereas it remained low (ratio of 2.5) in *miR-30b* milk-fed pups, leading to a significant difference ( $P < 0.05$ ) between the 2 groups. Furthermore, there was no longer any significant difference in *Claudin-3* and *Claudin-4* expression in the duodenum after PND-5.

PCA of pup duodenal proteome data showed a significant difference according to the type of milk at PND-5 (Figure 3A) but not at PND-15 (Figure 3B). At PND-5, the global analysis highlighted changes in 8 proteins out of 2213 analyzed in the duodenum of pups fed with *miR-30b* milk compared to WT ( $P < 0.001$ , corrected adjusted value  $< 0.10$ ; Table 1 and Supplemental Table 4). With *miR-30b* milk, the expression of the following 5 proteins decreased: retinol-binding protein 2 (Rbp2), alpha-1 catenin (Ctna1), apolipoprotein A-I (Apoa1), neutral ceramidase (Asah2), and negative elongation factor B (NelfB); and the expression of the following 3 proteins increased: histone H4 (H4), prolow-density lipoprotein receptor-related protein 1 (Lrp1), and isoform C of prelamin-A/C (Lmna). The functional analysis of these data using ingenuity pathway analysis (IPA,

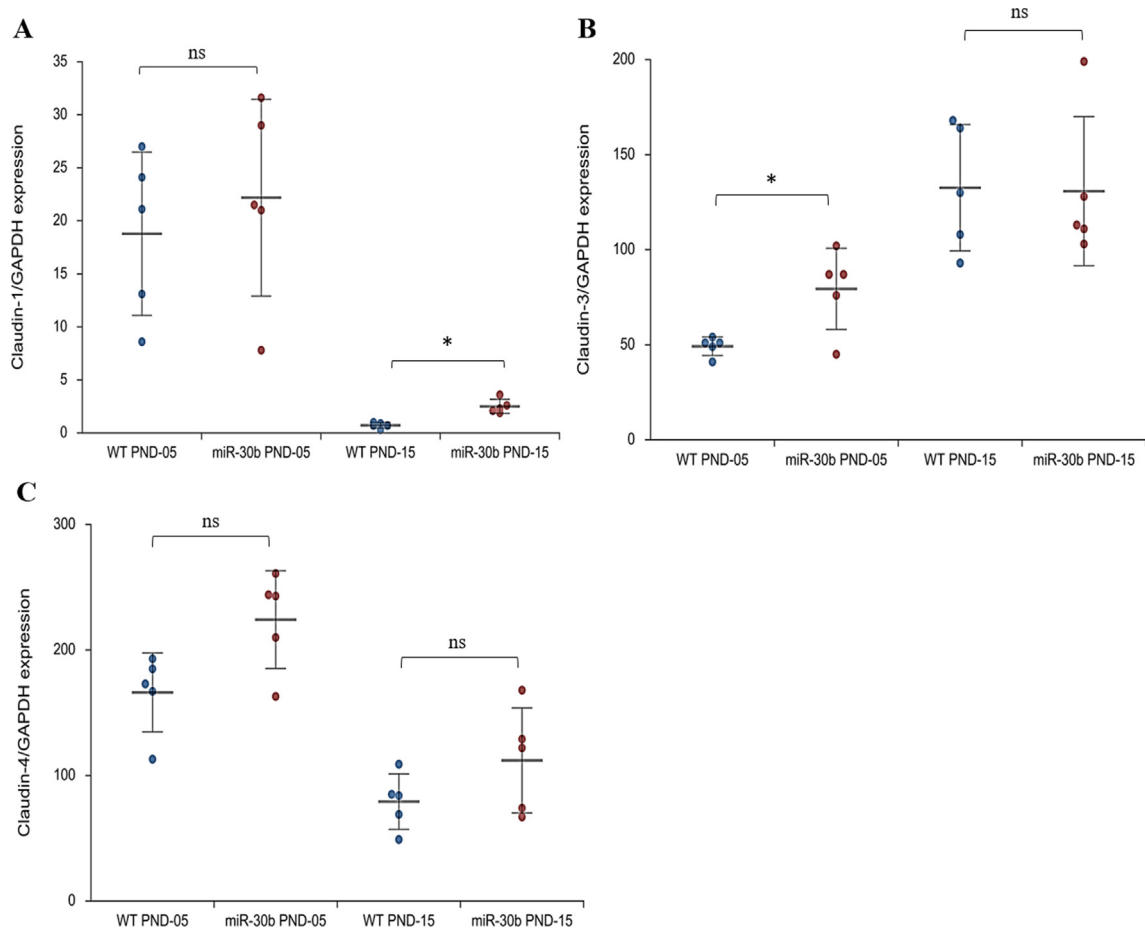
QIAGEN) highlighted that the biological pathways involved mainly concerned connective tissue development, morphology and function, lipid metabolism, and molecular transport (Table 2). When  $P < 0.05$  was considered, 3 additional proteins, filamin A (Flna), E-cadherin (Cdh1), and tight junction protein 1 (Tjp1, also known as ZO-1), which are essential for epithelial cell junctions, showed increased expression in the duodenum of pups fed with *miR-30b* milk compared to those fed with WT milk (Table 1). The permeability measurements on 6-d-old pups fed WT or *miR-30b* milk showed that the passage of both FSA and HRP through the duodenum was greater in pups fed WT milk compared with *miR-30b* milk, reaching a plateau after 45 min. The average measured postpassage FSA (Figure 4A) and HRP (Figure 4B) concentrations were 5700 ng/mL and 90 ng/mL, respectively, in WT pups and 183 ng/mL and 12 ng/mL, respectively, in pups fed *miR-30b* milk. These results revealed a reduced paracellular and transcellular permeability of the duodenum in pups fed *miR-30b* milk ( $P < 0.001$ ) at an early stage, which may be related to the increased expression of proteins essential for intestinal epithelial cell junctions before the intended intestinal closure.

### *miR-30b* milk displays minor protein, FAA, and total lipid changes

To determine which components in the milk may be associated with early intestinal maturation in the pups, the composition of



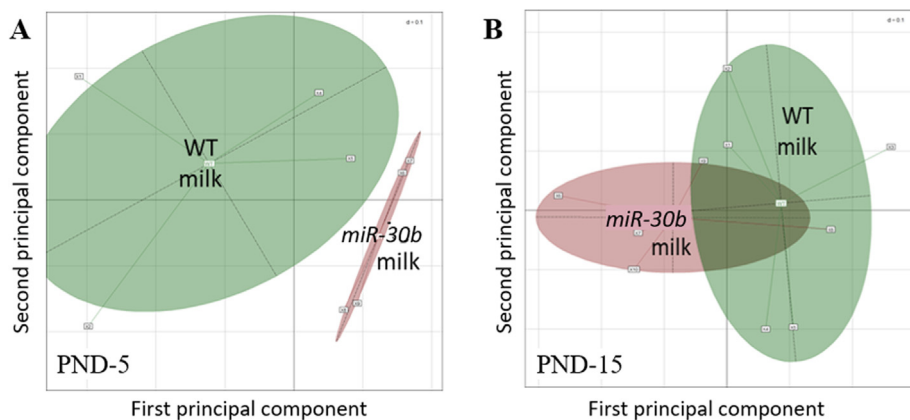
**FIGURE 1.** Histological analysis shows no significant structural changes in the duodenal tissue of pups fed *miR-30b* milk compared with pups fed control milk. Histological analysis of duodenal transverse sections from WT pups fed WT milk (A) or *miR-30b* milk (B) at PND-5 (HES), and from WT pups fed WT milk (C, E) or *miR-30b* milk (D, F) at PND-15 (HES and Alcian Blue-PAS staining). HES, hematoxylin and eosin; PAS, periodic acid-Schiff; PND, postnatal day; WT, wild-type.



**FIGURE 2.** Tight junction protein gene expression levels are increased in the duodenum of *miR-30b* milk-fed pups. *Claudin-1* (A), *Claudin-3* (B), and *Claudin-4* (C) transcriptomic expression levels in duodenal tissue at postnatal day (PND)-5 and PND-15 in wild-type (WT) pups fed WT or *miR-30b* milk. *P* values for comparison between *miR-30b* and WT groups were derived using the Mann–Whitney U test. Within a row, values followed by \* differ significantly ( $P < 0.05$ ); ns, nonsignificant.

the *miR-30b* milk was studied deeply at LD-15 around the lactation peak. Lactose and total protein levels did not differ between *miR-30b* and WT milk (data not shown); the composition of casein, whey acidic protein, and fatty acid synthase was also consistent (Supplemental Table 5). In contrast, the PCA score plot performed on the LC–MS/MS proteomic data showed a clear

separation between *miR-30b* milk and WT milk at LD-15 (Figure 5); out of 787 detected proteins, 9 were more abundant in the *miR-30b* milk ( $P < 0.10$ ; Table 3; Supplemental Table 5). These proteins were tenascin (Tena), osteopontin (Ospn), apolipoprotein C-I (ApoC1), cathepsin Z (Catz), pulmonary surfactant-associated protein D (SP-D), major urinary protein 2 (Mup2),



**FIGURE 3.** Duodenal proteome analysis shows significant differences according to the type of milk at postnatal day (PND)-5 but not at PND-15. Principal component analysis (PCA) of the duodenal proteome LC–MS/MS analysis of pups fed with wild-type (WT) or *miR-30b* milk at PND-5 (A) and PND-15 (B). LC–MS/MS, liquid chromatography tandem mass spectrometry.



**TABLE 1**  
Proteome modifications of the duodenal tissue of WT pups fed *miR-30b* milk<sup>1</sup>

Protein			WT milk feeding	<i>miR-30b</i> milk feeding	logFC	<i>P</i>	Adj. <i>P</i>
Decreased proteins in response to <i>miR-30b</i> milk	Rbp2	Retinol-binding protein 2	37.3 ± 7.1	26.8 ± 3.6	-0.84	5.43E-05	0.064
	Ctna1	Catenin alpha-1	11.8 ± 1.7	8.0 ± 1.2	-0.88	1.24E-04	0.064
	Apoa1	Apolipoprotein A-I	4.8 ± 1.7	2.3 ± 1.0	-1.36	1.74E-04	0.064
	Asah2	Neutral ceramidase	3.3 ± 0.5	0.8 ± 0.5	-2.05	8.48E-05	0.064
Increased proteins in response to <i>miR-30b</i> milk	NelfB	Negative elongation factor B	1.5 ± 0.6	0.0	-2.31	2.54E-04	0.070
	H4	Histone H4	2.8 ± 1.0	9.5 ± 4.0	1.24	1.51E-04	0.064
	Lrp1	Prolow-density lipoprotein receptor-related protein 1	1.8 ± 1.0	7.3 ± 1.0	1.50	1.64E-04	0.064
Essential proteins for epithelial cell junctions	Lmna	Isoform C of prelamin-A/C	0.0	2.3 ± 0.5	2.09	2.30E-04	0.070
	Flna	Filamin A	80 ± 18	124 ± 8	0.29	5.82E-04	0.14
	Tjp1	Tight junction protein-1	4.9 ± 2.9	9.3 ± 1.7	1.04	1.35E-02	0.33
	Cdh1	E-cadherin	1.8 ± 1.0	4.8 ± 1.3	0.94	1.52E-02	0.34

<sup>1</sup> Duodenal proteins showing differences based on LC-MS/MS analysis between pups fed WT and *miR-30b* milk at PND-5 (Limma Voom, *P* < 0.001, adjusted *P* < 0.10) and 3 proteins specifically involved in tight junctions (*P* < 0.05). LC-MS/MS, liquid chromatography tandem mass spectrometry; logFC, log fold change; PND, postnatal day; WT, wild-type.

glycosylation-dependent cell adhesion molecule 1 (Glc1), and serum amyloid proteins A-1 and A-2 (Saa1 and Saa2). IPA showed that these proteins are primarily involved in cell-cell signaling and interaction, cellular growth and proliferation, lipid metabolism, and tissue development (Table 4). In addition, the milk

concentration of free proteinogenic amino acids, which are highly digestible for newborns, was measured (Table 5). The concentration of one amino acid, proline, displayed a significant increase in *miR-30b* milk (140.6 ± 10.8 μM) compared with WT milk (106.1 ± 8.0 μM).

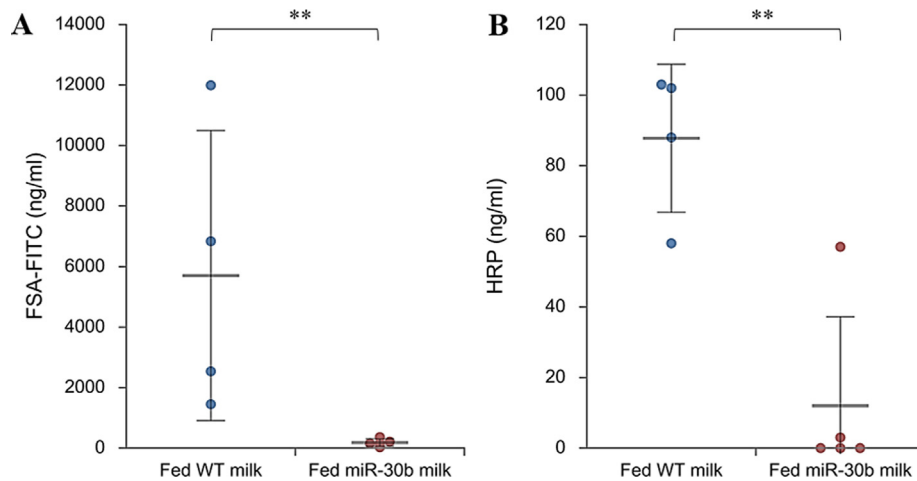
**TABLE 2**

Top biological functions related to modified proteins in duodenal tissue of pups breastfed with *miR-30b* milk at PND-5<sup>1</sup>

Biological functions		<i>P</i>	Molecules involved
Canonical pathways	Retinoate biosynthesis II	1.17E-03	—
	Acute phase response signaling	1.22E-03	—
	Osteoarthritis pathway	1.98E-03	—
	Ceramide degradation	2.05E-03	—
	Sphingosine and sphingosine-1-phosphate metabolism	2.64E-03	—
Physiological system development and function	Connective tissue development and function	3.18E-02–4.44E-05	Apoa1, NelfB, Lmna, Lrp1
	Tissue morphology	3.18E-02–4.44E-05	
	Hematological system development and function	2.87E-02–1.77E-05	Apoa1, Lrp1
	Immune cell trafficking	2.87E-02–1.77E-04	
Cardiovascular system development and function	2.64E-02–2.54E-04	Apoa1, Lmna, Lrp1	
Molecular and cellular functions	Lipid metabolism	3.24E-02–1.55E-06	
	Molecular transport	3.14E-02–1.55E-06	Apoa1, Asah2, Rbp2, Lmna, Lrp1
	Small molecule biochemistry	3.24E-02–1.55E-06	
	Vitamin and mineral metabolism	2.58E-02–2.21E-05	Apoa1, Ctna1, Lrp1
Cell signaling	3.04E-02–1.36E-04		

<sup>1</sup>Ingenuity pathway analysis, *P* < 0.05. PND, postnatal day.

<sup>1</sup> Ingenuity pathway analysis, *P* < 0.05. PND, postnatal day.



**FIGURE 4.** Paracellular and transcellular duodenal permeability are significantly reduced in pups fed *miR-30b* milk. (A) FSA and (B) HRP passage measurement through duodenal tissue of 6-d-old pups fed with wild-type (WT) or *miR-30b* milk in Ussing chambers at time point t-45 min of kinetics. *P* values for comparisons between transgenic and WT groups were derived using the Mann–Whitney U test. Within a row, values followed by \*\* differ significantly ( $P < 0.001$ ). FSA, fluorescein sulfonic acid; HRP, horseradish peroxidase.

The total lipid content was significantly higher in the *miR-30b* milk ( $219.0 \pm 16.4$  g/L) compared with WT milk ( $151.2 \pm 4.3$  g/L) ( $P < 0.05$ ) (Figure 6A), reinforcing our previous data [35], and a noticeable, significant increase in MUFAs and decrease in SFAs was observed in the *miR-30b* milk (24.7% and 50.3% for MUFA and SFA levels, respectively) compared with WT milk (17.2% and 59.1% for MUFA and SFA levels, respectively). Moreover, the analysis of sphingolipids showed a very significant increase in the total Cer concentration in *miR-30b* milk ( $17.6$   $\mu$ M) compared with WT milk ( $6.9$   $\mu$ M) ( $P < 0.05$ , Figures 6B and 6C)

with an associated increase in all classes of ceramides (Figure 6C) and specifically in 2 glucosyl-ceramides (hexoceramide C24:0 and lactoceramide C24:0) (2-fold higher). The total SM concentration was also significantly increased in *miR-30b* milk ( $163.7$   $\mu$ M) compared with WT milk ( $76.3$   $\mu$ M), likely due to an increase in several species of SMs (C16:0, C20:0, C24:0, C16:1, and C22:1) (Figure 6C).

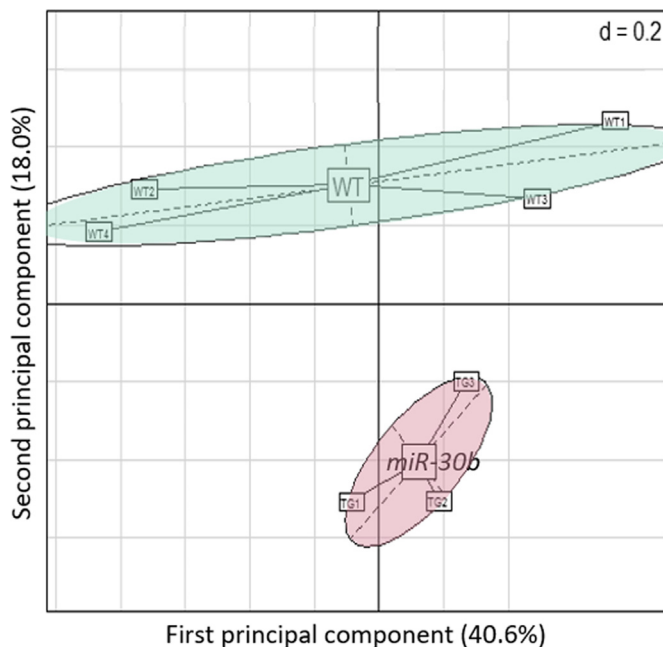
The average insulin concentration doubled in total *miR-30b* milk ( $1.88$  ng/mL) compared with WT milk ( $0.79$  ng/mL) ( $P < 0.01$ ) (Figure 7A). Although not significantly increased in total milk, the leptin concentration at LD-15 also showed an average 2-fold higher concentration in *miR-30b* skimmed milk ( $2.04$  ng/mL) than in WT skimmed milk ( $1.06$  ng/mL) ( $P < 0.05$ ) (Figure 7B). These differences in leptin concentrations that depend on the milk compartment were previously reported in human studies [2].

### Pups fed *miR-30b* milk show alterations in their adult growth trajectory

The early growth defect of pups suckling *miR-30b* milk compared to WT milk was observed from the third day of lactation (indicated by an asterisk in Figure 8), as previously reported [34]. This growth defect was maintained at weaning (day 28) with an average 3.8 g of individual difference, all sexes considered. Postweaning, within females and males, the differences in weight according to milk persisted from weaning to puberty (at approximately 6 and 8 wk old for females and males, respectively) (Figure 8).

This sexual dimorphism became significant in both WT and *miR-30b* milk feeding groups from day 33 and day 40, respectively, with males having higher average weights than females ( $P < 0.05$ ). On day 33, when nursed with the WT milk, males weighed 2.3 g more on average than females, and on day 40, when nursed with the *miR-30b* milk, males weighed 2.0 g more than females (Figure 8).

Finally, within groups of females and males, the difference between pups nursed with WT or *miR-30b* milk was not overcome in adulthood (through day 153); a difference of more than



**FIGURE 5.** Wild-type (WT) and *miR-30b* milk differ in their proteomic composition. Principal component analysis of the skimmed milk proteome LC–MS/MS analysis from WT and *miR-30b* transgenic mice at LD-15. LC–MS/MS, liquid chromatography tandem mass spectrometry; LD, lactation day.

**TABLE 3**  
*miR-30b* milk displays minor protein changes<sup>1</sup>

Protein		WT milk	<i>miR-30b</i> milk	logFC	P	Adj. P
Tena	Isoform 2 of tenascin	0.8 ± 1.0	8.3 ± 5.8	-2.82	2.45E-05	0.010
Ospn	Osteopontin	0.3 ± 0.5	5.0 ± 1.0	-2.85	2.54E-05	0.010
Catz	Cathepsin Z	0.8 ± 1.5	5.7 ± 0.6	-2.74	5.81E-05	0.015
SP-D	Pulmonary surfactant-associated protein D	1.3 ± 1.3	9.3 ± 3.1	-2.58	9.51E-05	0.019
Saa2	Serum amyloid A-2 protein	1.8 ± 2.4	10.3 ± 6.5	-2.59	0.00013	0.021
Mup2	Major urinary protein 2	0.8 ± 1.5	4.7 ± 1.5	-2.53	0.00032	0.042
Glc1	Glycosylation-dependent cell adhesion molecule 1	12.5 ± 1.7	34.7 ± 2.5	-1.31	0.00059	0.066
ApoC1	Apolipoprotein C-I	0.0	2.7 ± 0.6	-2.48	0.00083	0.075
Saa1	Serum amyloid A-1 protein	4.8 ± 5.6	13.7 ± 8.5	-2.26	0.00086	0.075

<sup>1</sup> Nine proteins show differences based on LC-MS/MS proteomic shotgun analysis between wild-type (WT) and *miR-30b* skimmed milk (Limma Voom,  $P < 0.001$ , adjusted  $P < 0.10$ ). LC-MS/MS, liquid chromatography tandem mass spectrometry; logFC, log fold change.

2 g in males and 1 g in females was maintained ( $P < 0.001$ ; Figure 8) despite comparable feed intake between groups (Supplemental Figure 1). Taken together, these results demonstrate that pups fed *miR-30b* milk show an early growth defect that persists into adulthood.

## Discussion

### Specific early intestinal phenotype of pups fed *miR-30b* milk

The structure and integrity of the duodenal tissue determine dietary nutrient absorption, which, in turn, contributes to driving pup development and growth [53,54]. In this study, pups (both

males and females) fed milk from transgenic mice overexpressing *miR-30b* in their mammary epithelial cells showed a particular phenotype in their duodenum, observed at PND-5, and this phenotype was associated with an early and persisting growth defect. In addition to reduced expression of *Ctna1*, a key protein of adherens junctions [55], the intestine of pups fed *miR-30b* milk presented an increase in several genes coding for proteins involved in gut barrier function and the regulation of tight junctions [29,56–58], such as Claudin-3 and Claudin-4, and genes involved in the formation and maintenance of epithelial cell junctions, such as *Tjp1*, *Cdh1*, and *Flna*. In addition, the pups' intestinal proteome showed higher expression of H4 and *Lmna*, an A-type intermediate filament protein that, together with B-type lamin, forms the nuclear lamina, a complex structure that

**TABLE 4**  
Top biological functions related to modified proteins in transgenic milk<sup>1</sup>

Biological functions	P	Molecules involved	
Molecular and cellular functions	Cell-to-cell signaling and interaction Cellular growth and proliferation Cellular movement Lipid metabolism Small molecule biochemistry	2.0E-02–3.8E-09 2.1E-02–2.6E-07 2.0E-02–5.4E-07 1.8E-02–1.0E-05 1.8E-03–1.0E-05	Catz, Glc1, Ospn, Saa1, SP-D, Tena Catz, Mup2, Ospn, Saa1, SP-D, Tena Catz, Ospn, Saa1, SP-D, Tena ApoC1, Saa1, SP-D, Ospn, Tena, Mup2
Physiological system development and function	Hematological system development and function Immune cell trafficking Tissue development Organ development Nervous system development and function	2.1E-02–4.5E-09 2.0E-02–2.4E-07 2.1E-02–2.6E-07 2.1E-02–2.6E-07 1.8E-03–2.6E-07	Catz, Glc1, Ospn, Saa1, SP-D, Tena Catz, Mup2, Ospn, Saa1, SP-D, Tena Catz, Mup2, Ospn, Tena

<sup>1</sup>Ingenuity pathway analysis,  $P < 0.05$ .

<sup>1</sup> Ingenuity pathway analysis,  $P < 0.05$ .

**TABLE 5**  
*miR-30b* milk displays an increase in proline concentration<sup>1</sup>

mean ± SEM (μM)	WT milk (n = 9)	<i>miR-30b</i> milk (n = 7)
Histidine	2.73 ± 0.49	2.28 ± 0.50
Isoleucine	11.80 ± 1.46	19.23 ± 6.80
Leucine	14.14 ± 1.79	21.47 ± 5.80
Lysine	128.1 ± 9.84	124.6 ± 9.07
Methionine	21.28 ± 3.06	15.14 ± 1.73
Cysteine	17.28 ± 2.33	14.59 ± 4.04
Threonine	127.1 ± 5.90	124.9 ± 12.42
Tryptophan	0.84 ± 0.24	0.86 ± 0.28
Phenylalanine	0.57 ± 0.06	0.53 ± 0.07
Valine	97.90 ± 6.58	100.6 ± 7.17
Arginine	33.60 ± 3.47	43.86 ± 7.04
Alanine	416.0 ± 51.24	556.0 ± 70.12
Aspartate	51.70 ± 4.45	71.47 ± 11.80
Asparagine	185.1 ± 24.30	265.70 ± 46.81
Glutamine	5.75 ± 1.97	6.96 ± 0.94
Glutamate	254.7 ± 14.54	285.8 ± 25.82
Proline	106.1 ± 8.0	140.6 ± 10.8 *
Glycine	354.5 ± 25.0	456.6 ± 46.17
Serine	246.9 ± 9.60	255.0 ± 22.18
Tyrosine	0.94 ± 0.17	1.11 ± 0.24
BCAAs	123.8 ± 6.33	141.3 ± 18.41
EAAAs	403.2 ± 17.78	427.8 ± 46.05
IAAs	437.9 ± 21.77	461.9 ± 73.97
SAAAs	38.80 ± 4.94	30.33 ± 7.47

<sup>1</sup> Concentrations of free proteinogenic amino acids in the milk of lactating wild-type (WT) mice and transgenic mice overexpressing *miR-30b* in the mammary gland at LD-15. Values are expressed as the mean ± SEM. \* represents a significant difference ( $P < 0.05$ ) in *miR-30b* milk compared with WT milk using a nonparametric Mann–Whitney test. BCAAs, branched-chain amino acids (isoleucine + leucine + valine); EAAAs, essential amino acids (phenylalanine + valine + threonine + tryptophan + methionine + leucine + isoleucine + lysine + histidine); IAAs, insulin-trophic amino acid (BCAAs + lysine + arginine + threonine); LD, lactation day; SAAAs, sulfur amino acids (methionine + cysteine); SEM, standard error of the mean.

contributes to nuclear structure maintenance [59]. Interestingly, these proteins, both involved in cell migration, proliferation, differentiation, and epigenetic regulation, were reported to be decreased in the small intestinal mucosa of intrauterine growth-retarded pig neonates associated with slower gut mucosa maturation [60,61]. The fact that Lmna and H4 were both increased in the intestine of pups fed with *miR-30b* milk may be consistent with changes in duodenal cells related to increased barrier maturation. Thus, taken together, these proteomic data point to a change in the maintenance and integrity of the duodenum of pups fed *miR-30b* milk with early modifications, such as an increase in tight and adherens junctions at PND-5, which is consistent with the highly reduced paracellular and transcellular permeability we measured compared with WT pups. These duodenal modifications were no longer visible once the barrier matured at approximately 2 wk [32]. These findings suggested an early maturation and closure of the intestinal barrier of the pups at PND-5 in response to ingesting milk because all studied pups were born from WT mothers and differed only by the milk provided by foster mothers (transgenic *miR-30b* compared with WT).

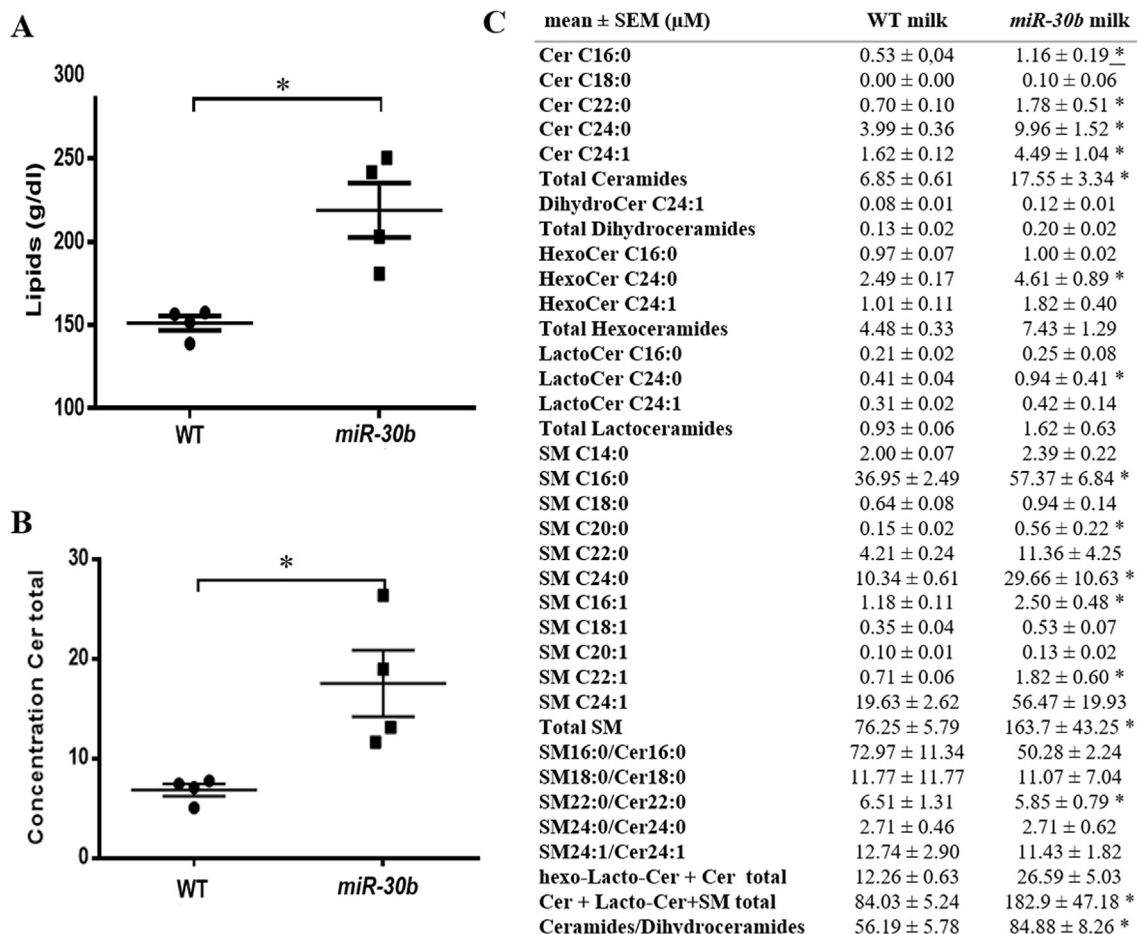
In addition, the intestinal proteome of pups fed *miR-30b* milk presented, at PND-5, an increase in Lrp1 expression, which mediates the uptake of ligands and molecules into cells for degradation [62] and regulates several important physiological processes (glucose homeostasis) and signaling pathways (leptin

or insulin signaling) in different tissues [63,64]. This finding was combined with reduced expression of Rbp2 [65,66] and Apoa1 [67], which are both involved in digestion, absorption, transport, and the metabolism of retinoic acid, an essential nutrient for development and growth [65] and the maintenance and integrity of intestinal epithelial cells [66]. Apoa1 is also implicated in the transport and assimilation of lipids [68,69]. Decreased expression was also observed for Asah2, an enzyme in the Cer pathway with a key role in the catabolism of dietary sphingolipids and, in turn, in the regulation of sphingolipid levels [70,71]. Altogether, these findings suggest early changes in the availability of trophic components (insulin) and bioactive lipids (such as sphingolipids) for intestinal integrity.

### Transgenic *miR-30b* milk composition changes associated with earlier intestinal maturation of the pups

We previously showed in our rodent model that the overexpression of *miR-30b* in the mammary epithelial cells led to a significant defect in lactation, changes in lipid droplet formation and secretion [35], and deregulation of 220 mammary genes, some being involved in secretion and transport of molecules [34]. This may explain changes in the *miR-30b* milk lipid and protein composition observed in the present study, such as changes in *Ospn*, *Saa1*, and *Saa2* expression. However, the precise molecular mechanisms remain to be explored. To understand which milk bioactive factors may be associated with early intestinal maturation, the milk composition was extensively analyzed. The components highlighted in this study present potential roles involved in 1) the modification of the development and integrity of the duodenal tissue, 2) the acceleration of barrier maturation, and 3) the alteration of the growth trajectory of the pups (Figure 9).

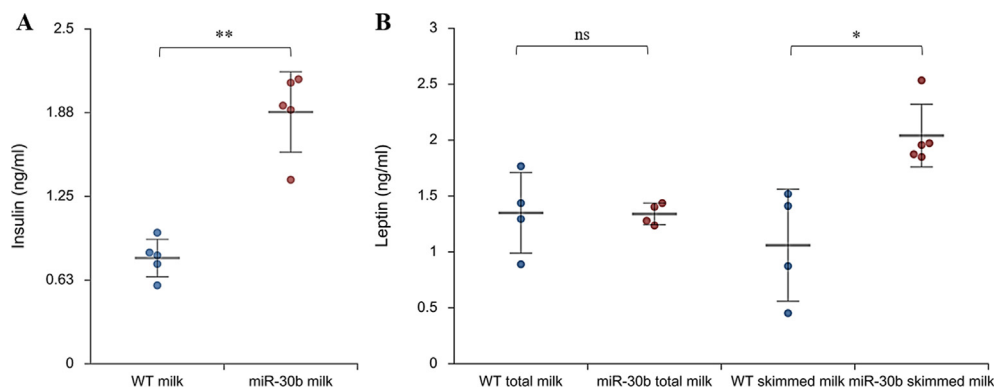
Modifications in duodenal tissue development and its absorption capacity [15,16,22] and in neonatal gut integrity and maturation [14,72–74] may result from the 2-fold increase in insulin, total ceramides, and SM concentrations combined with the enhanced (1.5-fold) total lipid content and the significant increase in MUFAs and decrease in SFAs in *miR-30b* milk compared with WT milk. Sphingolipids were reported to play crucial roles in maintaining barrier integrity, regulating nutrient absorption, and acting as signaling molecules to regulate the regeneration and differentiation of intestinal mucosa [75], likely through effectors such as cathepsin. Furthermore, the 6 increased minor proteins, ie, Tena, Catz, *Ospn*, *Glc1m1*, *Saa1*, and *Saa2*, are involved in connective tissue development and function and cell–cell interactions. More specifically, Tena is an extracellular matrix glycoprotein with milk levels associated with breastfeeding length [76–78], and it may have an impact on the development of pup gut epithelial tissues through interactions with cell surface receptors or modulation of other extracellular matrix proteins and cell–cell adhesion molecules [79]. Catz is a milk protease that retains activity within the infant’s stomach [80–82], and it may impact both cell surface and intracellular pathways through its involvement in the processing of cytokines and chemokines and its capacity to shed some extracellular receptors and cell adhesion molecules [83]. *Ospn* is highly present in milk [84–86], can maintain a bioactive form during gastrointestinal transit [87], and may affect pup gut tissue development by positively regulating intestinal cell proliferation, migration, and chemotaxis through



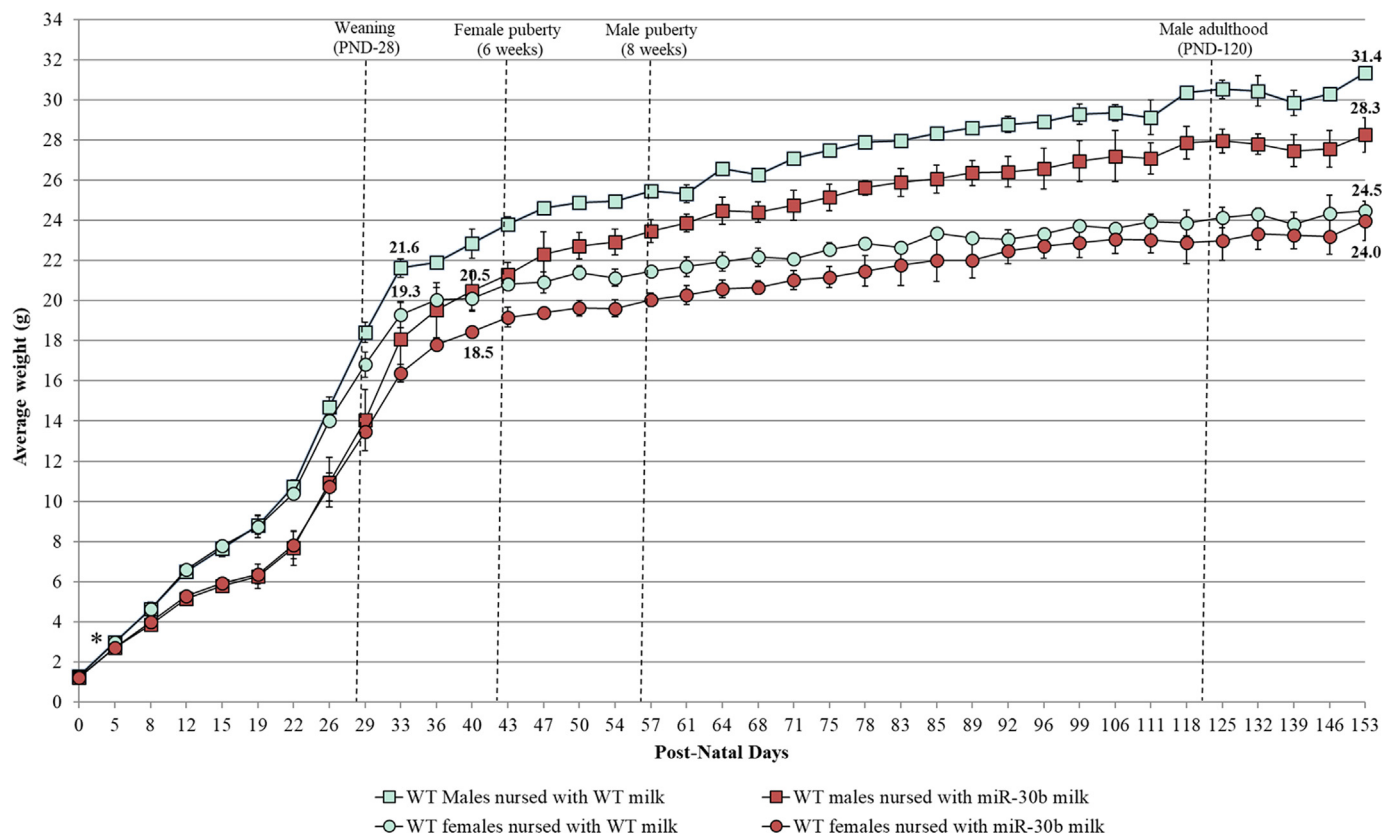
**FIGURE 6.** Total lipid, ceramide, and sphingolipid levels are significantly increased in *miR-30b* milk. Total lipid content (A), total ceramide (B), and sphingolipid (C) concentrations in wild-type (WT) and *miR-30b* milk at LD-15. *P* values for comparison between *miR-30b* and WT groups were derived using the Mann–Whitney U test. Within a row, values followed by \* or \*\* differ significantly ( $P < 0.05$  or  $P < 0.01$ , respectively). LD, lactation day.

binding to integrin receptors [88]. Glcm1 is a hormonally regulated milk glycoprotein linked to the milk mucin complex [89], and Glcm1's mammary form appears to be different from the endothelial classical form, leading to functions other than cell adhesion [89,90]. In particular, studies in cattle suggest that Glcm1 may play a role in mother-to-infant communication via the

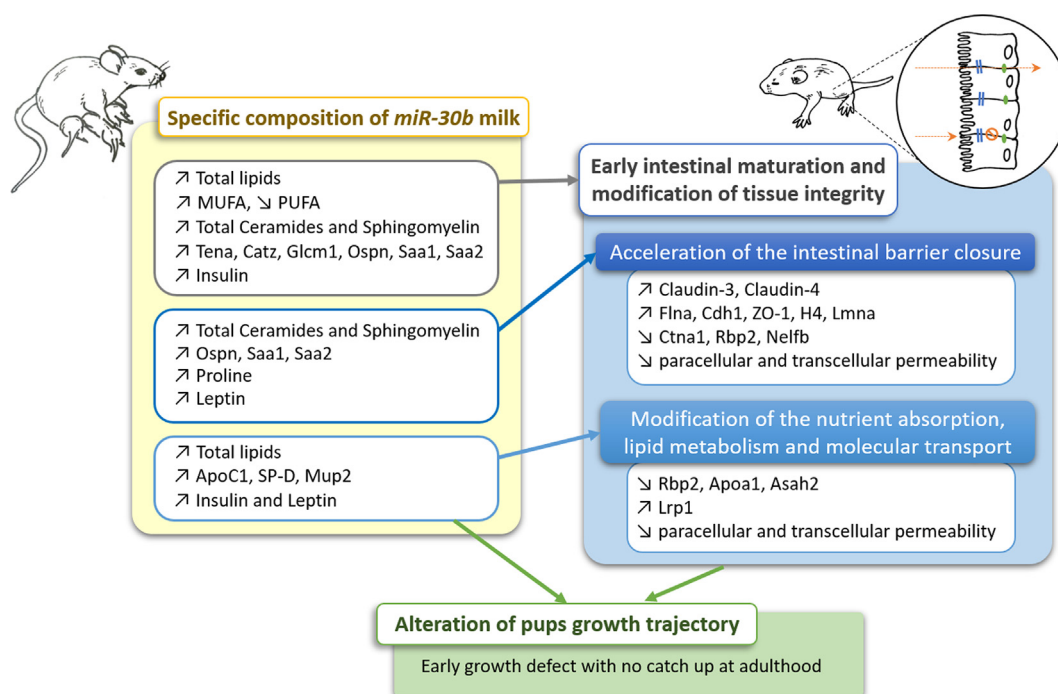
gastrointestinal tract, including functional differentiation and development [91]. Saa1 and Saa2 are primarily known as inducible acute phase proteins in response to injury, infection, and inflammation. In addition, recent studies have highlighted a role for Saa proteins, notably Saa1, in intestinal physiology during the early neonatal period [92]. For example, Saa isoforms in human



**FIGURE 7.** Insulin and leptin levels are significantly increased in *miR-30b* milk. Insulin (A) and leptin (B) levels in total or skimmed wild-type (WT) and *miR-30b* milk at LD-15. *P* values for comparison between *miR-30b* and WT groups were derived using the Mann–Whitney U test. Within a row, values followed by \* or \*\* differ significantly ( $P < 0.05$  or  $P < 0.01$ , respectively; ns: nonsignificant). LD, lactation day.



**FIGURE 8.** Mice nursed with *miR-30b* milk show alterations in their growth trajectory. Growth curves of wild-type (WT) pups nursed with WT (blank symbols) or *miR-30b* (dark symbols) milk from birth (PND-0) to adulthood (PND-153). Squares: males; Circles: females. An early growth defect observed from the third day of lactation is indicated with an asterisk. The growth curves show a significant difference ( $P < 0.001$ ) according to type of maternal milk, considering the sex effect, according to the statistical analysis of longitudinal data from PND-33 to PND-153 (linear mixed model with random animal effect and autoregressive correlation structure of order 1 to account for the correlation of the measurements over time). PND, postnatal day.



**FIGURE 9.** Illustration of the potential roles of *miR-30b* milk composition changes on the development and integrity of duodenal tissue, acceleration of barrier maturation, and alteration of pup growth trajectory.

colostrum were suggested to play an important role in both the development and maturation of intestinal defense and immune responses [93]. Thus, the intestinal phenotype of pups fed *miR-30b* milk may be due in part to the significant increase in these proteins in the milk.

The early maturation and closure of the intestinal barrier in *miR-30b* milk-fed pups may be elicited by some of the aforementioned molecules but also by FAAs and leptin, the levels of which are increased in *miR-30b* milk. Changes in MUFA and SFA levels in milk lead to alterations in mucus production and tight junction protein expression [16,23]. Particularly, the decrease in medium SFAs in *miR-30b* milk, such as capric (C10) and lauric (C12) acids, is commensurate with the earlier intestinal closure phenotype observed in our model, according to the previously reported C10- and C12-mediated enhancement of tight junction permeability in the intestines of rats and humans and in Caco-2 cells [94]. The increase in total ceramides and total SM concentrations in *miR-30b* milk can also promote the intestinal barrier function of pups [73,74]. SM supply through cow milk-based formula was associated with accelerated and precocious maturation in artificially reared suckling rats through a reduction in the permeability of the newborn intestine [95,96]. Studies in humans have shown that milk insulin and leptin are positively associated [9], which is consistent with the proportional increase in the levels of these 2 hormones in our study performed on *miR-30b* milk. The addition of insulin to preterm infant formulas or its oral supplementation results in an acceleration of intestinal maturation [24] and a reduction in intestinal permeability [97], respectively. Moreover, experiments on mice demonstrated that epithelial intestinal barrier function is reinforced by leptin by inducing the expression of tight junction proteins, i.e., ZO-1 and claudin-4 [25], which are upregulated in the intestinal proteome in pups fed *miR-30b* milk. Among the proteins with significantly increased expression in *miR-30b* milk, Ospn and both isoforms Saa1 and Saa2 also constitute important factors that may be involved in the precocious maturation and closure of the intestinal barrier. Ospn upregulates intestinal genes related to cytoskeletal structure, cell adhesion, and cell-cell communication, such as  $\beta$ -catenin and filamin A [98,99], and it improves intestinal barrier function [100,101]. Saa1 is able to promote intestinal epithelial wound sealing through the induction of cell spreading and increased epithelial cell attachment [102]. Finally, among the FAAs, proline levels increased in *miR-30b* milk. Proline is an important substrate for the synthesis of polyamines, which are essential for neonatal intestinal epithelial paracellular barrier function and remodeling [103, 104], notably by promoting E-cadherin expression at least partially through intracellular free  $\text{Ca}^{2+}$  concentration [105]. The oral supplementation of proline to neonatal piglets increases the expression of intestinal tight junction proteins and consequently improves the gut barrier [106]. The increased proline content in *miR-30b* milk could therefore contribute to the observed acceleration of intestinal maturation. The molecular mechanisms involved in the increased expression of tight junction proteins in relation to *miR-30b* milk composition require further elucidation. The expression of tight junction proteins can be regulated by a variety of signaling pathways, including the Ras-MAPK, Nrf2, and NF- $\kappa$ B pathways [107]. In particular, the MAPK signaling pathway, of which ceramides and leptin are important activators, is involved in the regulation of ZO-1

expression [108–110]. Thus, the significant increase of ceramides and leptin in *miR-30b* milk may be associated with the increase of ZO-1 expression observed in the duodenum of WT pups fed *miR-30b* milk. In addition, the effect of other milk components such as minerals and vitamins, not evaluated in this study, on duodenal maturation cannot be excluded. Indeed, higher levels of some minerals such as zinc lead to increased expression of ZO-1, via the activation of the PI3K/AKT/mTOR signaling pathway, and Claudin-3, leading to a strengthening of the epithelial barrier [94,111]. Furthermore, studies in rodents and cell culture have shown that an increase in dietary vitamin A and D content leads to an increase in the expression of ZO-1 and claudin-1 and so are associated with the maintenance and protection of the intestinal tight junction barrier [94,112,113]. Future investigations into these different components may provide further interesting insights into the possible mechanisms involved in this acceleration of barrier maturation.

The precocious closure of the intestinal barrier may impact the passage of milk components and nutrients by slowing their uptake and use or their bioavailability into the infant's circulation, with consequences on growth trajectory [3,32]. Studies have accordingly shown that even minor disturbances during neonatal gut barrier development or function can produce long-term effects on health [114,115].

Compositional changes in *miR-30b* milk implicate molecules involved in lipid metabolism and may play a role in neonatal growth, leading to growth defects. Notably, 3 increased proteins, ApoC1, SP-D, and Mup2, may impact lipid metabolism and their availability for gut neonates. This could be explained by the fact that ApoC1 inhibits the binding of  $\beta$ -VLDL to the Lrp protein [116] and negatively regulates lipoprotein lipase and phospholipase A2 [117–119], leading to impaired hydrolysis of milk lipoproteins and triglycerides, as reported in human *APOC1* transgenic mice [93–95]. This balance between ApoC1 and Lrp1 could modulate lipid availability to pups. The higher milk levels of the collectin protein SP-D may provide a beneficial effect on the gastrointestinal tract of pups due to its immune function in neonates [120,121]. The Mup lipocalin family, of which Mup2 is a member, plays an important role in nutrient metabolism, including serving as a metabolic signal to regulate glucose and lipid metabolism [122,123]. In addition, several reports indicate that milk leptin can play a role in neonatal growth [124–126]. Some studies specifically link lower leptin levels to increased postnatal weight and growth [9,10,127]. Thus, regarding our data, the increase in leptin in *miR-30b* milk and the growth defect observed in pups may be correlated. However, previous studies showed that the addition of insulin to preterm infant formulas allows better growth [14], which is not in line with the phenotype observed in pups fed *miR-30b* milk. Such discrepancies may result from the differences in birth timing (preterm compared with term) between the 2 studies. Moreover, concerning total lipids, their increase in *miR-30b* milk may have consequences for pup growth. Studies in humans have shown that higher total lipid content in breast milk was associated with lower infant growth velocity [128].

Our experiment was not specifically designed to aseptically collect sufficient maternal milk to ensure depth and precision of the description of milk microbial communities. However, it is important to be aware that alterations of various components in *miR-30b* milk, such as fatty acids, may shape the overall milk

environment and create the niche for a specific *miR-30b* milk microbiota [129] that can be vertically transferred to suckled pups [130,131]. This may have an impact on the infant gut colonization [132,133], and in turn, on tight junction remodeling and epithelial permeability through gut bacteria–epithelial cell interactions [134] and activation of the Nrf2 signaling pathway involved in the regulation of tight junction proteins [135,136]. Some studies have also shown a link between insulin and leptin levels in breast milk and the composition of the gut microbiota of the pups [137]. In our study, we cannot exclude an impact of high levels of insulin and leptin in *miR-30b* milk on the early duodenal maturation phenotype observed in pups in relation to a modification of their microbiota and bacterial endotoxin levels.

This study showed that feeding pups with modified milk, resulting from a single microRNA (*miR-30b*) deregulation in the mammary gland, can lead to long-term changes in the offspring with consequences on growth and intestinal physiology. Moreover, our data allowed the identification of subsequent variations in specific milk components that affect the maturation of the intestinal barrier of the pups and their growth, as summarized in Figure 9. Because of the outcomes reported, the implications of these findings from a mouse model may also be relevant and extend to other mammalian species, including humans (for example [58,96,98,106,115,126]). Some molecules were already partly qualified (such as fatty acids and sphingolipids, proline, Ospn, leptin, or insulin), but others have not been described for this specific role in vivo (such as Tena, ApoC1, SP-D, Mup2, Saa1, and Saa2). Because milk components can interact with each other and thus combine multifunctional roles impacting offspring growth and health [13], further investigations will be of interest to determine the mode of action of the molecules highlighted in this study with a focus on their putative interactions. In the future, the modulation of the content of milk bioactive components in infant diets could be of significant potential benefit for growth and health.

## Author Contributions

The authors' responsibilities were as follows—SLG, FLP: designed research; SLG, CCI, JL, JC, EA, CHB, CCh, SL, CH, AD, MCAG: conducted research; FJ, DL, SLG: analyzed data; SLG, MC, MCAG, FLP: wrote the paper; and all authors: read and approved the final manuscript.

## Conflict of Interest

The authors report no conflicts of interest.

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## Data Availability

The data that support the findings of this study are available in the methods of this article.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.tjnut.2023.08.003>.

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