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Human milk metabolites modulate gut barrier and immunity-related genes in an *in vitro* multicellular model of intestinal epithelium†

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Human milk (HM) is a complex food that meets nutritional newborn needs. The role of its bioactive components, particularly metabolites, in neonatal development remains poorly understood. This study focused on evaluating the effects of HM short chain fatty acids (SCFA), polyamines, tryptophan derivatives, gamma-aminobutyric acid (GABA), serotonin and lactate on several neonatal gut functions. The effects of these metabolites, at HM concentration, were analyzed individually or in mixture (MTB mix), on an *in vitro* multicellular model of intestinal epithelium, including Caco-2 (enterocytes), HT29-MTX (goblet cells), NCI-H716 (enteroendocrine cells) and M cells. Transcriptomic semi-screening revealed the impact of these metabolites, especially combined as a mixture, on various intestinal functions. MTB mix upregulated *CLDN3* and *CLDN4* while downregulating *CLDN1* and this was associated with a higher transepithelial resistance, highlighting its potential role in strengthening the intestinal epithelial barrier (IEB). MTB mix also reduced the expression of genes involved in mucus formation (*MUC1*, *TFF3*). Besides, MTB mix decreased immune-related gene expression (*CXCL8*, *MYD88*, *GPX2*), suggesting an immunomodulatory effect. Lastly, MTB mix decreased nutrient transporter and enzyme gene expression (*SLC2A1*, *SLC15A1*, *LCT*), suggesting that the mixture modulates digestive function. SCFA, especially butyrate, drove most of these effects, with a contribution from polyamines also, especially on IEB. Individually, GABA had a significant impact on all the examined functions, although these effects were absent with the MTB mix. Overall, this study highlights the ability of HM metabolites to modulate IEB and some genes related to the immune, digestive and endocrine functions *in vitro*, with some cumulative or attenuated effects when taken altogether *vs.* individually, emphasizing the importance of studying them as a mixture.

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Introduction

Human milk (HM) is a nutritive fluid that meets all the nutritional needs of newborns in the early postnatal period.[1] HM is associated with numerous health benefits, including a reduced risk of neonatal infections,[2] as well as growing evidence linking HM to a reduction in metabolic[3] and immune diseases.[4] These benefits are related to the dynamic and complex composition of HM. Indeed, HM is a complex food favouring the optimal development of the newborn, notably its immune and intestinal development, as well as the establishment of an optimal intestinal microbiota.[5] In addition to its nutritional components, HM contains several bioactive compounds that contribute to its health and developmental benefits.[1,6] These include bioactive proteins such as growth factors, hormones, cytokines, immunoglobulins and lactoferrin, a complex microbial fraction and numerous metabolites.[1,6] In particular, HM contains metabolites which are also present in the gut, due to the activity of the intestinal microbiota. These include short chain fatty acids (SCFA), lactate, polyamines (PA), tryptophan derivatives (TRPd) such as kynurenine, indole and indole-3-lactic acid (ILA), as well as gamma-aminobutyric acid (GABA) and serotonin,[7–11] which are known to play a key role in gastrointestinal physiology.

In the colon, SCFA, which include butyrate, acetate and propionate, are mainly derived from the microbial fermentation of non-digestible dietary fibers and absorbed by colonocytes to be used as energy sources.[12] Their ability to bind to various receptors such as free fatty acid receptor 2 and 3 (FFAR 2 and 3), as

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well as their action on histone deacetylase and other transcription factors, such as aryl hydrocarbon receptor (AHR), means that they exert numerous physiological actions in the intestine and they are also major players in the microbiota–gut–brain axis.[13,14] They are involved in lipid and carbohydrate metabolisms and help to maintain the intestinal epithelial barrier (IEB) by inducing mucus production and by enhancing the expression of tight junction proteins (TJP).[15,16] SCFA also moderate inflammation and promote immune tolerance.[17–19] Lactate, an organic acid, which is a product of glycolysis, can be used as an energy source, particularly by the intestinal microbiota, and enables the synthesis of SCFA.[20] PA, including spermine, spermidine and putrescine, promote the growth of the gastrointestinal tract[21] and enhance IEB by increasing expression of TJP.[22,23] They are also involved in the development of the immune system and have antioxidant properties.[22–24] TRPd, such as kynurenine and the various metabolites produced by the kynurenine pathway, which is the main tryptophan catabolic pathway (≈90%), have a complex relationship with the immune system and gut microbiota.[25] Other TRPd, indole and its derivatives such as ILA, produced by intestinal micro-organisms, enhance IEB by increasing TJP expression *in vitro* and *in vivo* and are able to modulate the host immune response.[26] GABA, the main inhibitory neurotransmitter in vertebrates, can be used or produced by the microbiota.[27] Its receptors are present on enterocytes, enteroendocrine cells and immune cells and it is involved in the regulation of various functions, including intestinal motility, immune and endocrine functions.[28] Serotonin, also known as 5-hydroxytryptamine (5-HT), is a neurotransmitter derived from tryptophan. It is involved in numerous gastrointestinal functions, notably motor and sensory functions. Recent studies have established a link between 5-HT, gut microbiota, and immune function.[29]

The origins of these metabolites within HM are not well understood, but it is likely that some are derived from maternal metabolism, particularly in the mammary gland.[30] It is also possible that some of these metabolites originate from the maternal intestinal microbiota *via* the bloodstream, or are produced by the HM resident microbiota.[31] A correlation was previously reported between putrescine concentration and *Gammaproteobacteria* abundance in the HM.[32] HM putrescine and butyrate were suggested to be of microbial origin, while most of the other metabolites targeted in the present study would be of endogenous maternal origin.[30,33]

While the role of the above-mentioned metabolites has been relatively well documented when they are produced in the gut or supplied by the diet in adults, their role in HM has been little explored. Only butyrate and PA have been studied through supplementation in infant formulas at HM concentrations. Butyrate strengthened the IEB, suppressed inflammatory reactions and could prevent food allergies, as evidenced in a mouse preclinical model and an *in vitro* human cell line of enterocytes (Caco-2).[17,19] PA supplementation during lactation in murine and porcine models promoted intestinal development, immune system and modulated the composition of the intestinal microbiota.[22,23,34,35] These interesting effects led us to explore further the role of HM metabolites. We hypothesized that, despite their low concentrations, HM metabolites could modulate several gut functions, either individually or when supplied together, as they are in HM.

In the present study, we addressed the impact of 12 metabolites present in HM, including SCFA, PA, TRPd as well as GABA, 5-HT and lactate, on the barrier, immune, digestive and endocrine gut functions using an *in vitro* multicellular model of intestinal epithelium. The model included Caco-2 enterocytes, HT29-MTX goblet cells, NCI-H716 enteroendocrine cells and M cells derived from Caco-2 differentiation. This multicellular model was composed of recognized and widely used cell lines of human origin, ensuring easier transferability to humans, and expressed genes present in infant gut such as lactase, ensuring also to a certain extent transferability to infants. This model was suitable for assessing the impact of several combinations of HM metabolites on the main intestinal functions using a semi-screening approach.[36,37] Metabolites were used at HM concentrations, either individually, combined by class, or as a mixture of all metabolites. This approach revealed the individual effects of these HM metabolites, as well as their combined effects, for a better assessment of their role in HM.

Materials and methods

Culture of the intestinal epithelial cells

The adherent intestinal Caco-2 cells (ECACC 09042001) and HT29-MTX cells (CRB CelluloNet; SFR Biosciences, CNRS UMS 3444, Inserm US 8, Université Claude-Bernard, Lyon, France) were routinely cultivated in high glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% heat inactivated fetal bovine serum (FBS) (HyClone Laboratories INC, South Logan, Utah, USA), 1% L-glutamine (Gibco) and 1% penicillin–streptomycin (Gibco) (hereafter referred as to complete DMEM). For the Caco-2 cells, complete DMEM was supplemented with 1% non-essential amino acid solution (Gibco). The floating intestinal NCI-H716 cells (ATCC CCL-251) and RAJI cells (ECACC 85011429) were routinely cultivated in Roswell Park Memorial Institute medium (RPMI-1640) (Gibco) supplemented with 10% heat inactivated FBS, 1% L-glutamine and 1% penicillin–streptomycin (hereafter referred as to complete RPMI). Each cell line was grown at 37 °C in a 5% CO_2 water-saturated atmosphere in 75 cm^2 flask (Falcon, Dutcher, Bernolsheim, France) and medium was completely changed for adherent cells and half changed for floating cells every 3 days. At 80% confluence, Caco-2 and HT29-MTX cells were treated with trypsin-EDTA (Gibco) and reseeded at 1–2 × 10^4 cell per cm^2 in flasks. The NCI-H716 and RAJI were reseeded 1 time per week at 1–2 × 10^4 cell per cm^2 in flasks.

Implementation of the multicellular model of intestinal epithelium

The multicellular model of intestinal epithelium was adapted from models previously developed[37–39] and included three

human colorectal adenocarcinoma (Caco-2, enterocyte cells model; HT29-MTX, goblet cells model; NCI-H716, enteroendocrine cells model) and M cells derived from differentiated Caco-2 cells with the action of RAJI cells. M cells are involved in the translocation of food and microbial antigens from the gut lumen to the lamina propria.[38]

Caco-2, HT29-MTX and NCI-H716 cells were seeded into inserts with 0.4 μm pore polyester membrane (Corning, Sigma-Aldrich, Saint Quentin Fallavier, France) coated with Matrigel (Corning) at 25 μg cm^{-2} and placed in a 24-well plate (Corning). A total of 2×10^5 cell per cm^2 (*i.e.* 6.6×10^4 cell per insert) was added in 250 μL of complete DMEM with a ratio of 8 Caco-2 cells for 1 HT29-MTX cell and 1 NCI-H716, in order to reproduce a functionally optimal model in the relative cell proportions of the small intestine,[40,41] and 1 mL of complete DMEM was added to the basal compartment. Medium of both compartments were changed every 3 days. After 15 days of co-culture, 1.6×10^5 RAJI cells, corresponding to a RAJI/initial Caco-2 cells ratio of 3 : 1, were added into the basal compartment to allow the differentiation of a part of Caco-2 cells into a M cell phenotype. The proportion of Caco-2 differentiated into M cells was evaluated to be ~7%,[39] *i.e.* close to the M cell ratio in human Peyer's patches (<5%).[42] Caco-2, HT29-MTX, NCI-H716 and RAJI cells were co-cultured for 6 days with a daily change of half of the DMEM media of the basal compartment after the first 3 days and a complete change of the media of the apical pole.

Stimulation of the multicellular model with the HM metabolites

At day 21, the model had reached maturity. RAJI cells were discarded after resuspension in the well by aspiration-reflux and the well was rinsed twice with Hanks balanced salt solution (HBSS, Gibco) supplemented with 1% penicillin–streptomycin (hereafter referred as to HBSS p/s) and once with DMEM Low Glucose (Gibco) supplemented with 1% L-glutamine and 1% penicillin–streptomycin (hereafter referred as to DMEM Low-Glu). At the apical compartment, the co-cultured Caco-2, HT29-MTX, NCI-H716 and M cells were then washed with DMEM Low-Glu. 250 μL and 1 mL of fresh DMEM Low-Glu were added at the apical and basal compartments, respectively, and the co-culture was incubated at 37 °C in a 5% CO_2 water-saturated atmosphere during 5 h to acclimatize cells to the new medium. Experiments are carried out in DMEM Low-Glu in order to achieve more physiological glucose concentrations and without FBS to avoid possible interference with lipid metabolites.[43] After acclimatization of the co-cultured Caco-2, HT29-MTX, NCI-H716 and M cell, targeted metabolites were added into the apical compartment at two different concentrations, corresponding to a concentration based on the mean and median HM concentrations of these metabolites as available in the literature (hereafter referred as to [HM]), and to the highest HM concentrations described in the literature (hereafter referred as to [HM+]). [HM] was based on the concentrations found in mature milk (Table 1 and ESI Table S1†). [HM+] concentration was 5-fold [HM], except for acetate and propionate where [HM+] concentration was 10-fold [HM], in order to cover the wide range of acetate concentrations found in HM. [HM+] concentration generally remained within the range of concentrations found in HM (ESI Table S1†). Metabolites were used individually, combined by classes (hereafter referred as to SCFA mix including butyrate, acetate, propionate, PA mix including spermine, spermidine, putrescine, and TRPd mix including indole, ILA, kynurenine), and as a mixture with all the metabolites (hereafter referred as to MTB mix). All metabolites were obtained from Sigma-Aldrich. 100× stock solutions of each metabolite were prepared in water, except for ILA which required 1.5% DMSO (Sigma-Aldrich), aliquoted and stored at −20 °C. Dilutions were then prepared in culture medium (complete DMEM Low-Glu). After 24 h of stimulation at 37 °C in a 5% CO_2 water-saturated atmosphere, the cells were lysed with lysis buffer (Buffer RLT, RNeasy Micro Kit, QIAGEN, Courtaboeuf, France), supplemented with 2% dithiothreitol at 2M (Sigma-Aldrich) and stored at −80 °C.

Measurements of epithelial permeability

The epithelial barrier integrity was evaluated by measuring the transepithelial electrical resistance (TEER) of the epithelial cell

Table 1 Concentrations of human milk metabolites tested in the multicellular model

Class	Metabolites	[HM][a]	[HM+][b]	No. CAS/references (Sigma-Aldrich)
Short chain fatty acid (SCFA)	Butyrate	0.75 mM	3.75 mM	107-92-6 /B103500
	Acetate	0.25 mM	2.5 mM	64-19-7/0070515
	Propionate[c]	0.25 mM	2.5 mM	79-09-4/P1386
Polyamines (PA)	Spermine	1 μM	5 μM	71-44-3/S4264
	Spermidine	1 μM	5 μM	124-20-9/S0266
	Putrescine	0.5 μM	2.5 μM	333-93-7/P5780
Tryptophan derivatives (TRPd)	Indole[d]	20 μM	100 μM	120-72-9/W259306
	Indole-3-lactic acid (ILA)[d]	20 μM	100 μM	832-97-3/I5508
	Kynurenine	200 nM	500 nM	2922-83-0/K8625
Non-proteinogenic amino acid	Gamma-aminobutyric acid (GABA)	120 nM	600 nM	56-12-2/A2129
Monoamine	Serotonin (5-HT)	120 nM	600 nM	153-98-0/H9523
Organic acids	Lactate	100 μM	500 μM	127-09-3/S2889

[a] [HM]: concentration based on mean and median concentrations of metabolites in human milk (HM). [b] [HM+]: high concentration in HM, corresponds to 5- to 10-fold the [HM]. [c] Propionate concentrations are poorly documented in HM and we aligned it with acetate concentration. [d] The concentrations used correspond to an estimate based on those found in the intestines and feces of newborn, as data from HM were not available when the study began. A recent study measured indole and ILA in HM at concentrations lower that those used in the present study.[11]

layer before (T-5) and after 5 h (T0) DMEM Low-Glu acclimatization, and after 24 h metabolites stimulation (T24), with STX2-plus/EVOM3 devices (WPI). The culture plates were placed on a plate at 37 °C for 5 min before the measurements to stabilize TEER. TEER was expressed as a percentage difference in Ohm × cm^2 between T0 and T24, all relative to control wells not exposed to the metabolites, according to the following calculation: delta $TEER_{T24-T0}$/delta $TEER_{T24-T0,\ CTRL}$.

Paracellular permeability was assessed using Lucifer yellow, a small fluorescent marker (452 Da). After 24 h metabolite stimulation, supernatants were discarded and apical and basal compartments were washed with HBSS p/s. Protected from light, 250 µL of HBSS p/s supplemented with Lucifer yellow at a final concentration of 200 µM (Sigma-Aldrich) were added to the apical compartment and 1 mL of HBSS p/s was added to the basal compartment. Culture plates were placed at 37 °C in a 5% CO_2 water-saturated atmosphere during 2 h. Basal supernatants were sampled and Lucifer yellow's fluorescence was measured using spectrometer (FLUOstar Omega, BMG LABTECH, Champigny s/Marne, France). Paracellular permeability to Lucifer yellow was assessed by calculating the percentage of basal pole fluorescence over Lucifer yellow's fluorescence at 200 µM, all relative to the control wells not exposed to the metabolites, according to the following calculation: [(LYbasal compartment − blank)/(LY 200 µM − blank) × (100)]/[(LYbasal compartment − blank)/(LY 200 µM − blank) × (100)]$_{CTRL}$.

Measurement of gene expression

Total RNA of the cell lysis was extracted using an RNA purification kit (RNeasy Micro Kit, QIAGEN), following manufacturer instructions. 1 µg of RNA was reverse transcribed into cDNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Fisher Scientific, Illkirch, France) following the protocol provided by the manufacturer. The high-throughput qPCR amplification was performed using the Takara SmartChip Real-time PCR system by the EcogenO Platform (OSUR, Université de Rennes, France) on 5184-well plates. Amplification conditions were as follows: 95 °C for 5 min of denaturation, followed by 45 cycles of 10 s of denaturation at 95 °C, 30 s of annealing at 60 °C and 30 s of elongation at 72 °C, and a final cycle of denaturation–annealing. The melting curves of each product were analyzed to assess amplification specificity. qPCR reactions were performed in duplicate and a threshold cycle (C_t) of 30 was used as the detection limit. The list of genes performed is detailed in ESI Table S2.† The expression of targeted genes was calculated as $2^{-\Delta\Delta CT}$ after normalization to the mean expression of at least three of the household genes (*ACTB*, *B2M*, *HPRT1*, *RPLP0*, *RPL18*, *RPL37A* and *TBP*; ΔC_t) (ESI Table S2†) and to the mean expression of targeted genes of controls (wells without metabolites) ($\Delta\Delta C_t$).

Statistical analysis

Experiments were conducted in three to four runs on two to four replicates. Gene expression and epithelial permeability were analyzed by two-way ANOVA (treatment and run as factors) followed by Tukey test on GraphPad Prims Statistical Software (v9.3.1) software. P values < 0.05 were considered significant and P value < 0.1 considered as a tendency.

The multivariate statistical analyses were performed using R Statistical Software (v4.2.3; R Core Team 2021). Principal component analysis (PCA) was used for separating metabolites based on their profile with FactoMineR package. 33 variables were used: gene expression and TEER measurements.

Results

HM metabolites affected expression of genes related to the barrier, immune, digestive and endocrine intestinal functions depending on the metabolite class

A PCA was carried out considering all the dataset (expression of 32 genes associated to barrier, immune, digestive and endocrine intestinal functions as well as TEER, a physiological marker of intestinal barrier) (ESI Tables S3–S5†). These genes were selected because they are representative of functions whose establishment is crucial during the early postnatal period (ESI Table S2†). PCA was performed separately in [HM] and [HM+] conditions (ESI Fig. S1† and Fig. 1). [HM] corresponded to the mean or median concentrations of these metabolites in HM, and [HM+] corresponded to their high concentrations found in HM (Table 1). The [HM+] concentration remained within the range of concentrations found in HM. Overall, concentrations corresponded to those found in mature milk (ESI Table S1†). PCA labelling was done with regard to the metabolite class, *i.e.* SCFA, PA, TRPd, whereas GABA, 5-HT and lactate were labelled individually as they could not be included into a metabolite class. A separate label was attributed to the mixture of all metabolites (MTB mix). Although all groups were not clearly separated, different patterns were somewhat revealed with [HM+] on the first component of the PCA, explaining 15.74% of the differences, as illustrated by the distinct barycenters and confidence intervals for each metabolite class (Fig. 1A). Of note, separation between metabolite classes was hardly visible with [HM] (ESI Fig. S1†). At [HM+], the scatter plot highlighted that the SCFA group and the MTB mix stood out clearly from all the other groups on component 1 (Fig. 1A). These two groups were positively associated with the gene expression of solute carrier family 16 member 1 (*SLC16A1*) encoding for a transporter of SCFA, intestinal alkaline phosphatase (*ALPI*) involved in lipopolysaccharide (LPS) detoxification and a marker of maturation of the multicellular model, and TEER, associated with the ion transepithelial permeability (Fig. 1B). The TRPd group and GABA were rather on the other side of the component 1 of the PCA, positively associated with the gene expression of the immune marker myeloid differentiation primary response gene (*MYD88*), as well as with the tight junction genes claudin 1 (*CLDN1*), occludin (*OCLN*) and tight junction protein 1 (*TJP1*) on components 1–2 (Fig. 1). PA group was separated from the other groups on component 2, especially SCFA, GABA and

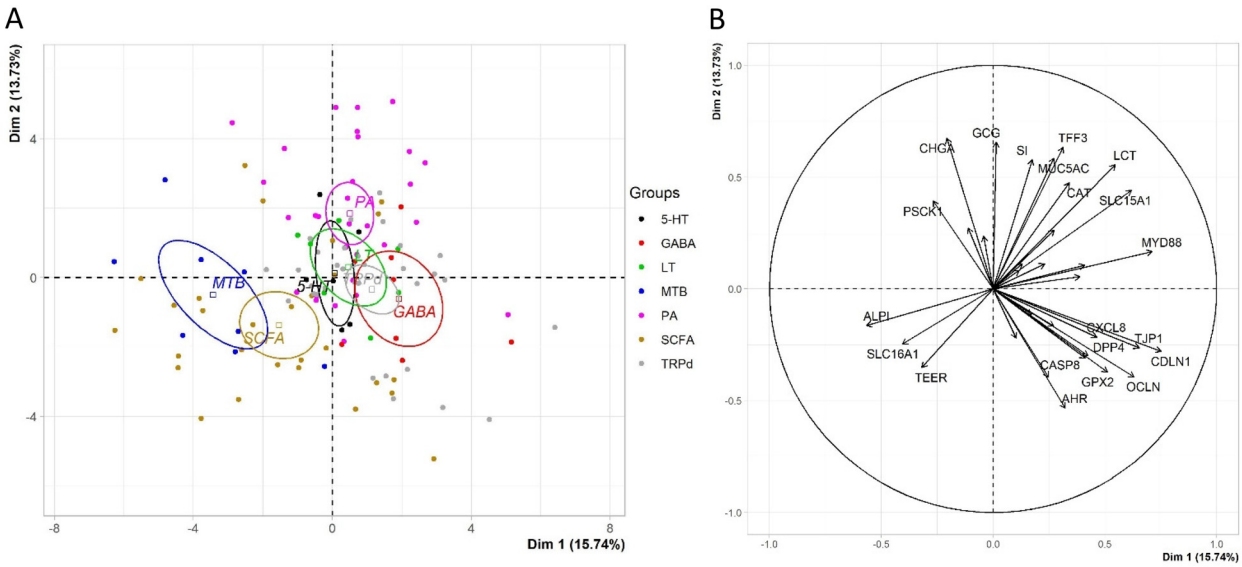

Fig. 1 Contrasted impact of human milk metabolites used at human milk high concentrations on the barrier, immune, digestive and endocrine functions in a multicellular model of intestinal epithelium. Principal component analysis was performed on 32 variables corresponding to the expression of genes involved in various intestinal functions (refer to ESI Table S2†) and 1 variable corresponding to the transepithelial electrical resistance (TEER), a marker of *para*- and *trans*-epithelial permeability. (A) Scatter plot of individuals on dimension 1 (Dim 1) and 2 (Dim 2), with the confidence circles (corresponding to the confidence interval of the estimate of the barycenter's coordinates) and the barycenters of 7 groups defined according to their metabolic class: SCFA (butyrate, acetate, propionate, and SCFA mix), PA (spermine, spermidine, putrescine and PA mix), TRPd (kynurenine, indole, indole-3-lactic acid and TRPd mix), GABA, serotonin, lactate and the mixture of all the metabolites (MTB). (B) Correlation circle for Dim 1 and 2, with the 21 most involved variables represented. 5-HT: serotonin; GABA: gamma-aminobutyric acid; LT: lactate; SCFA: short chain fatty acid; PA: polyamines; TRPd: tryptophan derivatives.

TRPd groups (Fig. 1A) and was positively associated with the expression of two genes related to the intestinal endocrine function, chromogranin A (*CHGA*) and proglucagon (*GCG*), as well as with the digestive enzyme genes sucrase isomaltase (*SI*) and lactase (*LCT*) and the mucus-forming genes mucin 5AC (*MUC5AC*) and trefoil factor 3 (*TFF3*) (Fig. 1B). Finally, lactate and 5-HT were central into the PCA, indicating poor impact (Fig. 1A). Overall, this PCA underlined that the different metabolite classes found in HM differentially modulated genes associated with the barrier, immune, digestive and endocrine gut functions, as detailed below.

HM metabolites modulated the intestinal barrier function

HM metabolites were also analyzed individually against the control, *i.e.* culture medium alone. These analyses highlighted major effects on intestinal barrier function, in particular for SCFA. TEER was indeed increased with butyrate ($P < 0.05$) and SCFA mix ($P < 0.01$) (Table 2 and Fig. 2A). This was corroborated by a decrease in the paracellular diffusion rate of Lucifer yellow with SCFA mix at [HM+] ($P < 0.01$) (Fig. 2B).

Butyrate and SCFA mix significantly altered expression of genes that mediate tight and adherent junctions. Relative to control cells, expression of claudin 3 (*CLDN3*) and E-cadherin (*CDH1*) were greater with butyrate ($P < 0.05$) and SCFA mix ($P < 0.01$) respectively, and expression levels of *CLDN1* and *TJP1* were lower in butyrate ($P < 0.05$ and <0.1 respectively) and SCFA mix ($P < 0.0001$ and <0.01 respectively) (Table 2 and ESI Table S3†). Most changes in tight and adherent junction protein expressions were observed at the [HM+] with, sometimes, intermediate or significant effects at [HM] (for *CLDN1* and *CLDN3* for instance). SCFA also impacted the expression of genes encoding mucins. Butyrate increased the expression of *MUC1* ($P < 0.05$), but the SCFA mix decreased the expression of both *MUC1* ($P < 0.05$) and *MUC5AC* ($P < 0.01$). Butyrate and SCFA mix decreased *TFF3* expression, as early as [HM] for the SCFA mix ($P < 0.01$ and <0.0001 respectively). Regarding the trophic factors, SCFA decreased the expression of the *Ki-67* proliferation marker both individually (acetate, propionate and butyrate, $P < 0.0001$, <0.01 and <0.05 respectively) and in combination (SCFA mix, $P < 0.05$) at [HM+]. The SCFA mix also induced a decrease of caspase 8 (*CASP8*, $P < 0.05$) (Table 2).

Some changes in the expression of genes related to the barrier function were also observed with the other metabolites, such as PA that modulated the expression of TJP genes. Indeed, the individual PA spermine, spermidine and putrescine increased expression of claudin 4 (*CLDN4*, $P < 0.01$, <0.05 and <0.05 respectively), observable from the [HM]. This was not the case with the PA mix, which conversely decreased the expression of other TJP genes such as *CLDN1* and *TJP1*, from the [HM] ($P < 0.05$) (Table 2). In addition, PA individually and in combination, decreased the expression of *CASP8* ($P < 0.01$). Unlike with SCFA and PA mix, GABA and ILA increased the expression of *TJP1* at [HM+] ($P < 0.05$). GABA decreased claudin 7 (*CLDN7*) expression at the two concentrations tested

Table 2 Relative expression of genes related to barrier function in the multicellular model of intestinal epithelium differentially expressed after exposure to metabolites at human milk concentrations

Metabolite	Function	Protein name/physiological marker	*Gene*/Code[a]	CTRL[b]	[HM][b]	[HM+][b]	*p*-Value
SCFA mix	Permeability	Cadherin 1	*CDH1*	1.03 ± 0.05^{a}	1.03 ± 0.06^{a}	1.22 ± 0.04^{b}	0.010
		Claudin 1	*CLDN1*	0.96 ± 0.06^{a}	0.85 ± 0.05^{b}	0.55 ± 0.02^{c}	<0.0001
		Tight junction protein 1	*TJP1*	1.01 ± 0.07^{a}	1.03 ± 0.08^{a}	0.84 ± 0.06^{b}	0.005
		trans-Epithelial electric resistance	TEER	1.00 ± 0.05^{a}	1.17 ± 0.05^{b}	1.19 ± 0.05^{b}	0.009
	Mucus	Mucin 1	*MUC1*	1.06 ± 0.19^{a}	1.19 ± 0.10^{a}	0.70 ± 0.08^{b}	0.014
		Mucin 5AC	*MUC5AC*	1.03 ± 0.11^{a}	0.92 ± 0.10^{ab}	0.56 ± 0.05^{b}	0.007
		Trefoil factor 3	*TFF3*	1.01 ± 0.09^{a}	0.80 ± 0.07^{b}	0.48 ± 0.03^{c}	<0.0001
	Trophism	Antigen Kiel 67	*Ki-67*	1.05 ± 0.15^{a}	0.97 ± 0.10^{ab}	0.77 ± 0.13^{b}	0.018
		Caspase 8	*CASP8*	1.01 ± 0.06^{a}	0.85 ± 0.06^{ab}	0.69 ± 0.07^{b}	0.033
Butyrate	Permeability	Claudin 1	*CLDN1*	1.02 ± 0.08$^{\#}$	1.05 ± 0.05$^{\#}$	0.81 ± 0.07$^{\#\#}$	0.041
		Claudin 3	*CLDN3*	1.05 ± 0.12^{a}	1.10 ± 0.22^{ab}	1.54 ± 0.25^{b}	0.023
		trans-Epithelial electric resistance	TEER	1.00 ± 0.03^{a}	0.99 ± 0.02^{a}	1.18 ± 0.05^{b}	0.005
	Mucus	Mucin 1	*MUC1*	1.08 ± 0.23^{a}	2.15 ± 0.36^{ab}	2.12 ± 0.33^{b}	0.017
		Trefoil factor 3	*TFF3*	1.04 ± 0.12^{a}	1.01 ± 0.15^{a}	0.55 ± 0.06^{b}	0.003
	Trophism	Antigen Kiel 67	*Ki-67*	1.01 ± 0.05^{a}	0.90 ± 0.09^{a}	0.55 ± 0.05^{b}	<0.0001
Acetate	Trophism	Antigen Kiel 67	*Ki-67*	1.01 ± 0.07^{a}	0.99 ± 0.05^{a}	0.78 ± 0.06^{b}	0.002
Propionate	Trophism	Antigen Kiel 67	*Ki-67*	1.01 ± 0.05^{a}	0.93 ± 0.03^{ab}	0.84 ± 0.04^{b}	0.048
PA mix	Permeability	Claudin 1	*CLDN1*	1.00 ± 0.08^{a}	0.80 ± 0.06^{b}	0.79 ± 0.04^{b}	0.016
		Tight junction protein 1	*TJP1*	1.01 ± 0.09$^{\#}$	0.81 ± 0.06$^{\#\#}$	0.83 ± 0.07$^{\#\#}$	0.044
	Trophism	Caspase 8	*CASP8*	1.02 ± 0.07^{a}	0.73 ± 0.06^{b}	0.70 ± 0.04^{b}	0.004
Spermine	Permeability	Claudin 4	*CLDN4*	1.02 ± 0.08^{a}	1.60 ± 0.10^{b}	1.42 ± 0.20^{b}	0.002
	Trophism	Caspase 8	*CASP8*	1.05 ± 0.17^{a}	0.84 ± 0.06^{b}	0.83 ± 0.05^{b}	0.011
Spermidine	Permeability	Claudin 4	*CLDN4*	1.01 ± 0.06^{a}	1.15 ± 0.10^{ab}	1.25 ± 0.10^{b}	0.045
	Trophism	Caspase 8	*CASP8*	1.05 ± 0.15^{a}	0.86 ± 0.09^{b}	0.81 ± 0.06^{b}	0.013
Putrescine	Permeability	Claudin 4	*CLDN4*	1.02 ± 0.08^{a}	1.44 ± 0.14^{b}	1.26 ± 0.07^{b}	0.002
	Trophism	Caspase 8	*CASP8*	1.02 ± 0.10^{a}	0.91 ± 0.08^{ab}	0.76 ± 0.07^{b}	0.050
GABA	Permeability	Claudin 7	*CLDN7*	1.00 ± 0.04^{a}	0.87 ± 0.06^{b}	0.87 ± 0.03^{b}	0.024
		Tight junction protein 1	*TJP1*	1.01 ± 0.07^{a}	1.19 ± 0.07^{a}	1.31 ± 0.08^{b}	0.048
	Trophism	Caspase 3	*CASP3*	1.02 ± 0.10^{a}	1.38 ± 0.09^{b}	1.47 ± 0.09^{b}	0.002
5-HT	Permeability	Claudin 4	*CLDN4*	1.02 ± 0.09^{a}	0.82 ± 0.13^{ab}	0.76 ± 0.06^{b}	0.030
Kynurenine	Permeability	Claudin 1	*CLDN1*	1.01 ± 0.05^{a}	1.10 ± 0.10^{ab}	1.25 ± 0.09^{b}	0.027
		Occludin	*OCLN*	1.01 ± 0.04^{a}	1.06 ± 0.06^{ab}	1.19 ± 0.06^{b}	0.008
ILA	Permeability	Tight junction protein 1	*TJP1*	1.00 ± 0.06^{a}	1.13 ± 0.06^{ab}	1.20 ± 0.06^{b}	0.030
	Trophism	Caspase 8	*CASP8*	1.00 ± 0.03^{a}	1.09 ± 0.10^{ab}	1.26 ± 0.07^{b}	0.038

[a] Gene names are in italics; code refers to physiological marker. [b] Results presented were obtained by RT-qPCR (mean ± SEM). CTRL refers to control cells not exposed to metabolites and [HM] and [HM+] refer to cells treated with metabolites or groups of metabolites indicated at the mean or high concentrations found in human milk, respectively. SCFA mix includes butyrate, acetate and propionate and PA mix includes spermine, spermidine and putrescine. Differences between groups were assessed by two-way ANOVA. ^{a,b,c} different letters indicate that groups differ significantly (p-value < 0.05) and $^{\#,\#\#}$ symbols indicate that groups tend to differ (p-value < 0.1). 5-HT: serotonin; GABA: gamma-aminobutyric acid; ILA: indole-3-lactid acid; PA: Polyamines; SCFA: short chain fatty acid.

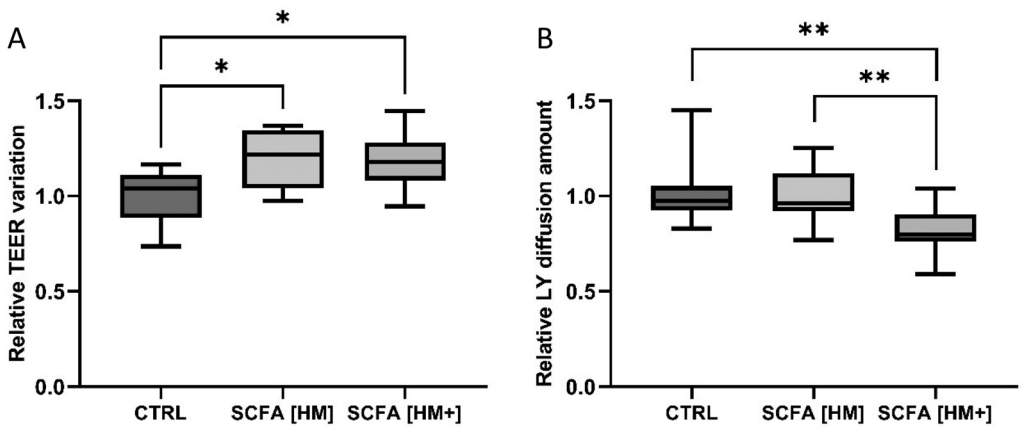

Fig. 2 Effect of the short chain fatty acid mix at human milk concentrations on transepithelial electrical resistance and paracellular permeability to Lucifer yellow in a multicellular model of intestinal epithelium. The multicellular model of intestinal epithelium was exposed to short chain fatty acids (SCFA, acetate, propionate and butyrate) for 24h at [HM] and [HM+], with the CTRL not supplemented with SCFA. (A) Relative transepithelial electrical resistance (TEER) was expressed as the difference in Ohm × cm^2 between TEER at T24 and T0, relative to this difference in CTRL conditions. (B) Lucifer yellow diffusion was measured in the basal pole 2h after it was added to the apical pole and was expressed relative to the fluorescence of Lucifer yellow at 200 µM and to the CTRL condition. Statistical: two-way ANOVA. *: $P < 0.05$; **: $P < 0.01$.

($P < 0.05$). Besides, GABA and ILA increased the expression of *CASP3* and *CASP8* ($P < 0.05$) respectively, from [HM] for GABA (Table 2). Finally, at [HM+], 5-HT decreased *CLDN4* expression ($P < 0.05$) and kynurenine increased *CLDN1* ($P < 0.05$) and *OCLN* ($P < 0.01$) expression (Table 2).

MTB mix recapitulated the effects of the individual metabolites or classes of metabolites, such as the increase in TEER at [HM+] ($P < 0.01$) observed with SCFA, the decrease in *CLDN1* ($P < 0.01$) observed with SCFA and PA mix, the decrease in *MUC1* ($P < 0.05$) and *MUC5AC* ($P < 0.05$) observed with SCFA, and the increase ($P < 0.05$) in *CLDN3* and *CLDN4* induced by butyrate and the individual PA respectively (Fig. 3A). MTB mix had no significant impact on proliferation (*Ki-67*) and apoptosis genes (*CASP3* and *8*, ESI Table S3†).

HM metabolites, especially SCFA, affected the expression of genes involved in immunity

Butyrate decreased the expression of *MYD88* ($P < 0.05$) and increased that of *ALPI* ($P < 0.0001$) and *AHR* ($P < 0.01$) at the [HM+]. Propionate also increased *AHR* expression, although this gene expression was not modulated by the SCFA mix. Acetate decreased ($P < 0.05$) glutathione peroxidase 1 (*GPX1*) and chemokine C–X–C motif ligand 8 (*CXCL8*) expression at [HM] and [HM+] respectively. When combined, the SCFA also regulated *ALPI* ($P < 0.0001$), *CXCL8* ($P < 0.01$) and *MYD88* ($P < 0.01$), with effects from [HM] for the last one. Finally, SCFA mix also led to a reduction in the expression of transforming growth factor beta (*TGFB1*) ($P < 0.05$) and glutathione peroxidase 2 (*GPX2*) ($P < 0.0001$) (Table 3). Likewise, GABA had an impact on immune markers increasing the expression of *CXCL8* ($P < 0.05$) and *AHR* ($P < 0.05$) and decreasing that of *TGFB1* ($P < 0.05$), with effects from the [HM] for *AHR* and *TGFB1* (Table 3).

The other metabolites tested hardly affected the expression of immune markers, especially PA, whose impact was limited to a higher *GPX1* expression with spermine. TRPd, such as ILA and kynurenine increased *AHR* expression at [HM] and [HM+] respectively, and ILA decreased cyclooxygenase-2 (*COX-2*) expression (Table 3).

Most of the regulations observed previously were also found after exposure to the MTB mix, especially those induced by SCFA mix, including lower expression of *CXCL8* ($P < 0.001$), *MYD88* ($P < 0.05$), *GPX2* ($P < 0.0001$) and higher expression of *ALPI* ($P < 0.05$) (Fig. 3B).

HM metabolites modulated the expression of genes involved in digestive and endocrine functions

SCFA had a significant impact on markers of digestive function, especially associated to carbohydrate and peptide transport, as well as to endocrine function. Firstly, solute carrier family 2 member 1 (*SLC2A1*), encoding glucose transporter type 1 (GLUT1), was negatively affected by SCFA mix ($P < 0.05$), GABA ($P < 0.05$), 5-HT ($P < 0.0001$) and lactate ($P < 0.01$), whereas *SLC16A1*, encoding monocarboxylate transporter 1 (MCT1), was positively affected ($P < 0.05$) by SCFA mix and butyrate. Regarding peptide transport, solute carrier family 15 member 1 (*SLC15A1*), encoding peptide transporter 1 (PEPT1), showed a higher expression with PA mix ($P < 0.05$), putrescine ($P < 0.05$) and GABA ($P < 0.01$) and a lower expression with SCFA mix ($P < 0.01$), whereas *ANPEP*, encoding alanyl aminopeptidase, was increased with SCFA mix ($P < 0.01$), butyrate ($P < 0.01$) and kynurenine ($P < 0.05$) (Table 4). These changes in expression were mostly observed at [HM+] with intermediate expression for [HM]. Additional digestive enzymes were affected. *LCT*, whose expression tended to be reduced with butyrate, was significantly reduced with the SCFA mix at [HM+] ($P < 0.0001$) (ESI Table S5† and Table 4). In addition, GABA and 5-HT increased the expression of *SI* at [HM+] ($P < 0.05$) (Table 4).

MTB mix decreased the expression of *SCL2A1* ($P < 0.05$) similar to the response to SCFA mix, GABA, 5-HT and lactate, and decreased ($P < 0.01$) the expression of *SLC15A1* and *LCT* as observed with the SCFA mix as well. Surprisingly, MTB mix decreased *SI* expression at [HM], while it was higher with GABA and 5-HT at [HM+] (Fig. 3C).

Finally, some genes related to the endocrine function were affected by HM metabolites, including a lower expression of the proglucagon encoding gene (*GCG*) with SCFA mix at both concentrations ($P < 0.05$) and an increase in the endocrine marker chromogranin A (*CHGA*) with MTB mix at the [HM+] ($P < 0.05$) (Table 4 and Fig. 3C).

Discussion

HM is a nutritive fluid with a complex and dynamic structure and composition.[1] It contains numerous classes of metabolites, often at very low concentrations compared to concentrations found, for example, in the colon.[44,45] The role of these metabolites has been little studied in a breastfeeding context, either alone or even more as a combination of different metabolic classes, as it occurs naturally in HM. Here, we investigated whether HM metabolites, at the concentrations found in HM, could modify the expression of genes associated with different intestinal functions, alone and especially in combination. For this purpose, we used an original and complex multicellular model of the intestinal epithelium, including cells mimicking enterocytes, goblet cells, enteroendocrine cells and M cells, which expressed genes representative of the barrier, immune and endocrine intestinal functions, also in infancy.

Our study revealed that several HM metabolites affected the expression of genes associated with barrier, immune, digestive and endocrine functions in a multicellular model of intestinal epithelium. The impacts of the HM metabolites on these gene expressions indicate that HM metabolites could potentially be bioactive in the newborn during breastfeeding, and could therefore modulate and influence the different gut functions, which are under maturation in infancy. In general, the metabolites had an effect at the [HM+], although some statistical effects were also observed at [HM]. Although [HM+] corresponds to metabolite concentrations 5 or 10-fold higher than

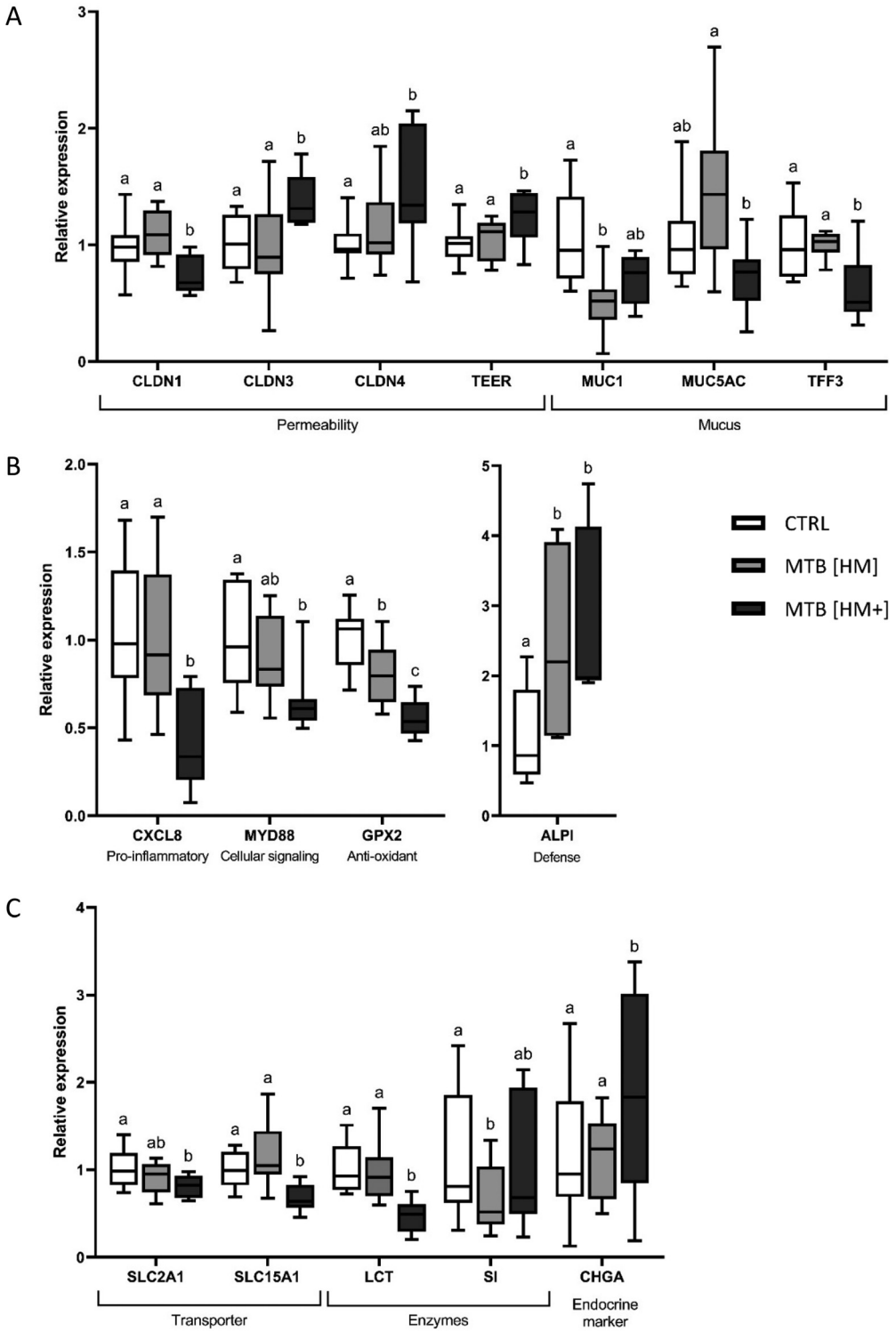

Fig. 3 Relative expression of genes related to different intestinal functions in a multicellular model of intestinal epithelium after exposure to the mix of human milk metabolites. The multicellular model of intestinal epithelium was exposed to the mix of metabolites (butyrate, acetate, propionate, spermine, spermidine, putrescine, gamma-aminobutyric acid, indole, indole-3-lactic acid, kynurenine, serotonin, lactate) for 24h at human milk mean or median concentrations (MTB [HM]) and high human milk concentrations (MTB [HM+]), or to culture medium without metabolites (CTRL). (A) Differentially expressed barrier function genes. Relative transepithelial electrical resistance (TEER) was expressed as the difference in Ohm × cm^2 between TEER at T24 and T0, relative to this difference in CTRL conditions. (B) Differentially expressed immune function genes. (C) Differentially expressed digestives and endocrine function genes. Statistical: two-way ANOVA. ^{a,b,c} different letters indicate that groups differ significantly (p-value < 0.05).

Table 3 Relative expression of genes related to immune function in the multicellular model of intestinal epithelium differentially expressed after exposure to metabolites at human milk concentrations

Metabolite	Function	Protein name	Gene	CTRL[a]	[HM][a]	[HM+][a]	*p*-Value
SCFA mix	Anti-inflammatory	Transforming growth factor beta 1	TGFB1	1.00 ± 0.05^{a}	0.98 ± 0.04^{ab}	0.87 ± 0.04^{b}	0.050
	Pro-inflammatory	Chemokine C–X–C motif ligand 8	CXCL8	1.02 ± 0.10^{a}	1.12 ± 0.08^{a}	0.70 ± 0.06^{b}	0.007
	Receptor/cellular signaling	Myeloid differentiation primary response gene	MYD88	1.02 ± 0.09^{a}	0.79 ± 0.09^{b}	0.62 ± 0.04^{b}	0.003
	Anti-oxidant	Glutathione peroxidase 2	GPX2	1.00 ± 0.07^{a}	1.02 ± 0.06^{a}	0.56 ± 0.03^{b}	<0.0001
	Defense	Intestinal alkaline phosphatase	ALPI	1.08 ± 0.15^{a}	1.48 ± 0.16^{a}	2.94 ± 0.20^{b}	<0.0001
Butyrate	Receptor/cellular signaling	Myeloid differentiation primary response gene	MYD88	1.03 ± 0.09^{a}	0.92 ± 0.08^{ab}	0.73 ± 0.05^{b}	0.034
		Aryl hydrocarbon receptor	AHR	1.02 ± 0.09^{a}	1.17 ± 0.09^{ab}	1.35 ± 0.06^{b}	0.003
	Defense	Intestinal alkaline phosphatase	ALPI	1.04 ± 0.12^{a}	0.82 ± 0.08^{a}	2.44 ± 0.29^{b}	<0.0001
Acetate	Pro-inflammatory	Chemokine C–X–C motif ligand 8	CXCL8	1.04 ± 0.11^{a}	0.94 ± 0.19^{ab}	0.61 ± 0.07^{b}	0.044
	Anti-oxidant	Glutathione peroxidase 1	GPX1	1.14 ± 0.23^{a}	0.69 ± 0.08^{b}	0.99 ± 0.08^{ab}	0.013
Propionate	Receptor/cellular signaling	Aryl hydrocarbon receptor	AHR	1.02 ± 0.09^{a}	1.09 ± 0.05^{a}	1.27 ± 0.04^{b}	0.011
Spermine	Anti-oxidant	Glutathione peroxidase 1	GPX1	1.10 ± 0.20^{a}	0.70 ± 0.09^{ab}	0.63 ± 0.08^{b}	0.026
GABA	Anti-inflammatory	Transforming growth factor beta 1	TGFB1	1.01 ± 0.05^{a}	0.88 ± 0.06^{b}	0.87 ± 0.05^{b}	0.020
	Pro-inflammatory	Chemokine C–X–C motif ligand 8	CXCL8	1.02 ± 0.07^{a}	1.10 ± 0.16^{ab}	1.32 ± 0.11^{b}	0.029
	Receptor/cellular signaling	Aryl hydrocarbon receptor	AHR	1.00 ± 0.07^{a}	1.32 ± 0.05^{b}	1.31 ± 0.09^{b}	0.015
Kynurenine	Receptor/cellular signaling	Aryl hydrocarbon receptor	AHR	1.02 ± 0.07^{a}	1.21 ± 0.15^{ab}	1.34 ± 0.12^{b}	0.004
ILA	Pro-inflammatory	Cyclooxygenase-2	COX-2	1.00 ± 0.03^{a}	1.08 ± 0.03^{a}	0.86 ± 0.04^{b}	0.001
	Receptor/cellular signaling	Aryl hydrocarbon receptor	AHR	1.00 ± 0.06^{a}	1.25 ± 0.10^{b}	1.18 ± 0.07^{ab}	0.050

[a] Results presented were obtained by RT-qPCR (mean ± SEM). CTRL refers to control cells not exposed to metabolites and [HM] and [HM+] refer to cells treated with metabolites or groups of metabolites indicated at the mean or high concentrations found in human milk, respectively. SCFA mix includes butyrate, acetate and propionate. Differences between groups were assessed by two-way ANOVA. ^{a,b} different letters indicates that groups differ significantly (p-value < 0.05). GABA: gamma-aminobutyric acid; ILA: indole-3-lactid acid; SCFA: short chain fatty acid.

Table 4 Relative expression of genes related to digestive and endocrine functions in the multicellular model of intestinal epithelium differentially expressed after exposure to metabolites at human milk concentrations

Metabolite	Function	Protein name	Gene	CTRL[a]	[HM][a]	[HM+][a]	*p*-Value
SCFA mix	Nutrient transporter	Glucose transporter type 1	SLC2A1	1.01 ± 0.06^{a}	1.03 ± 0.07^{a}	0.81 ± 0.03^{b}	0.011
		Monocarboxylate Transporter 1	SLC16A1	1.05 ± 0.18^{a}	1.42 ± 0.43^{a}	2.60 ± 0.39^{b}	0.017
		Peptide transporter	SLC15A1	1.01 ± 0.06^{a}	0.94 ± 0.19^{a}	0.85 ± 0.08^{b}	0.005
	Digestive enzyme	Alanyl aminopeptidase	ANPEP	1.01 ± 0.09^{a}	1.41 ± 0.08^{b}	1.34 ± 0.12^{b}	0.010
		Lactase	LCT	1.03 ± 0.11^{a}	0.98 ± 0.06^{a}	0.54 ± 0.03^{b}	<0.0001
	Endocrine	Proglucagon	GCG	1.15 ± 0.29^{a}	0.56 ± 0.13^{b}	0.51 ± 0.10^{b}	0.019
Butyrate	Nutrient transporter	Monocarboxylate transporter 1	SLC16A1	1.09 ± 0.18^{a}	1.20 ± 0.15^{a}	1.66 ± 0.11^{b}	0.013
	Digestive enzyme	Alanyl aminopeptidase	ANPEP	1.10 ± 0.19^{a}	1.10 ± 0.17^{a}	1.64 ± 0.18^{b}	0.003
PA mix	Nutrient transporter	Peptide transporter 1	SLC15A1	1.01 ± 0.08^{a}	1.05 ± 0.07^{ab}	1.34 ± 0.10^{b}	0.029
Putrescine	Nutrient transporter	Peptide transporter 1	SLC15A1	1.02 ± 0.13^{a}	1.10 ± 0.11^{ab}	1.55 ± 0.18^{b}	0.043
GABA	Nutrient transporter	Glucose transporter type 1	SLC2A1	1.01 ± 0.04^{a}	0.88 ± 0.07^{b}	0.89 ± 0.05^{b}	0.026
		Peptide transporter 1	SLC15A1	1.00 ± 0.04^{a}	1.10 ± 0.06^{ab}	1.28 ± 0.07^{b}	0.004
	Digestive enzyme	Sucrase isomaltase	SI	1.04 ± 0.12^{a}	1.48 ± 0.14^{ab}	1.69 ± 0.18^{b}	0.047
5-HT	Nutrient transporter	Glucose transporter type 1	SLC2A1	1.01 ± 0.06^{a}	0.73 ± 0.05^{b}	0.84 ± 0.06^{b}	<0.0001
	Digestive enzyme	Sucrase isomaltase	SI	0.95 ± 0.11^{a}	1.42 ± 0.15^{ab}	1.52 ± 0.14^{b}	0.025
Lactate	Nutrient transporter	Glucose transporter type 1	SLC2A1	1.01 ± 0.06^{a}	0.84 ± 0.04^{b}	0.90 ± 0.06^{b}	0.002
Kynurenine	Digestive enzyme	Alaniyl aminopeptidase	ANPEP	1.01 ± 0.06^{a}	1.14 ± 0.08^{ab}	1.27 ± 0.08^{b}	0.049

[a] Results presented were obtained by RT-qPCR (mean ± SEM). CTRL refers to control cells not exposed to metabolites and [HM] and [HM+] refer to cells treated with metabolites or groups of metabolites indicated at the mean or high concentrations found in human milk, respectively. SCFA mix includes butyrate, acetate and propionate and PA mix includes spermine, spermidine and putrescine. Differences between groups were assessed by two-way ANOVA. ^{a,b} different letters indicate that groups differ significantly (p-value < 0.05). GABA: gamma-aminobutyric acid; PA: polyamines; SCFA: short chain fatty acid.

their mean\median HM concentrations, it remains within a concentration range that can be found in HM (ESI Table S1†) and could therefore be physiologically relevant in newborns. Multivariate PCA analysis, a non-supervised analysis, highlighted contrasting effect of metabolites, depending on their class, with notably a separation between SCFA, PA and GABA/TRPd effects. Interestingly, despite different and possibly opposite effects of the different classes of metabolites, the PCA

analysis highlighted that the MTB mix displayed the most contrasting effect compared to all the other metabolites, either individually or in mixtures by classes. Notably, the effects of the MTB mix were close to those of the SCFA mix, suggesting that the effects of the MTB mix were mainly driven by SCFA.

The MTB mix affected the barrier function of the multicellular intestinal epithelium model at [HM+] by increasing TEER as well as by modulating TJP and mucin gene expressions despite sometimes different effects of individual metabolites on TJP gene expression. Expression of *CLDN3* and *4* was increased with the MTB mix, as with butyrate and individual PA, respectively. On the contrary, *CLDN1* was decreased with the MTB mix, as with the SCFA mix and PA mix, despite increased expression with kynurenine. Thus, the action of SCFA and/or PA prevailed on that of kynurenine over *CLDN1* expression. SCFA and PA mix decreased *TJP1* expression, whereas GABA and ILA increased it. As a result, the MTB mix had no effect. Of note, TEER increased with the MTB mix at [HM+] only, while it increased from [HM] with SCFA, suggesting that different effects of the different metabolites may occur individually or within the MTB mix. The effects of SCFA on intestinal permeability have been widely documented in the literature, and there is a consensus that they strengthen the IEB both *in vitro* and *in vivo*,[16,19,46] as found in the present study with measurements of TEER and Lucifer yellow permeability. SCFA have been reported to increase the expression of tight and adherent junction,[44,46,47] as we found in our study with *CLDN3* and *CDH1* respectively. However, exposure to SCFA also decreased the expression of other TJP in our study, such as *CLDN1* and *TJP1*, as previously described in *in vitro* models.[47,48] These opposite effects of SCFA on different tight and adherent junction gene expression underline the complexity and important dynamics of tight and adherent junction genes under the influence of SCFA. Furthermore, a study carried out in Caco-2 cells showed that the action of SCFA did not only involve regulation of TJP expression, but also a more efficient recruitment of TJP to the cell junctions, mediated by the activation of AMP-activated protein kinase.[16,49] PA modulated TJP expression without impacting TEER, with differential effects when added individually (increased *CLDN4*) or in mixture (decreased *CLDN1* and *TJP1*). *In vitro*, PA depletion studies highlighted their role in the expression of TJP such as *OCLN* and *TJP1*.[50] In a pig model, PA supplementation increased TJP gene expression.[22,23] The effects of PA on intestinal permeability are largely dependent on their concentration, with permeabilizing effects at concentrations above 15 mM.[51,52] In our study, concentrations were in the μM range and it is difficult to conclude on the functional effect of PA, as TEER was not affected. However, the increase in *CLDN4* expression and the decrease in *CLDN1* expression, also present with the MTB mix, highlight their potential effects on the IEB. Overall, despite a combination of differential effects on TJP expression, the MTB mix seemed to strengthen IEB *in vitro* because the TEER was increased in the conditions we tested. The effect seemed driven by SCFA and PA because the effects observed in the MTB mix were also found in the latter.

Mucins, which are transmembrane or secreted glycoproteins, are also essential components of the IEB as they form a protective mucus layer. *MUC1*, a gene highly expressed in HT29-MTX cells, decreased with the MTB mix only at [HM], while *MUC5AC*, a secreted mucin, was higher at [HM] than at [HM+], but did not differ from the control. Mucin genes are finely regulated and extremely sensitive to the environment such as nutritional environment, bacterial products or cytokines.[15,53] For instance, these modulations could be due to a transient effect of SCFA. Indeed, butyrate increased *MUC1* expression, but SCFA mix reduced both *MUC1* and *MUC5AC*. SCFA are known to modulate mucus production in both *in vitro* and *in vivo* studies.[15,19,54] A previous study has also shown that SCFA mixtures can decrease *MUC5AC* expression, though at higher acetate and propionate concentrations than the ones we used.[15] Finally, *TFF3*, which encodes a peptide involved in intestinal repair, decreased with the MTB mix, SCFA mix, and butyrate, consistent with previous studies showing butyrate ability to reduce *TFF3* expression.[55]

Regarding trophicity, the MTB mix did not affect genes related to proliferation or apoptosis. However, SCFA mix, PA mix, GABA and ILA modulated some of these markers. SCFA, especially butyrate, has been shown to have an antiproliferative effect on cancer cells by reducing the Ki-67 marker.[56,57] Likewise, most of these metabolites reportedly affect caspase expression.[58–62] Thus, in the MTB mix, possible interferences between metabolites could occur allowing fine regulation of trophicity.

In terms of intestinal immune markers, the MTB mix overall decreased their expression, with, notably, a decrease in *CXCL8* which encodes the pro-inflammatory cytokine interleukin 8 (IL-8), *MYD88* involved in toll like receptor (TLR) signal transduction, *GPX2* involved in scavenging oxygen free radicals, suggesting downregulation of inflammation by the MTB mix. These effects occurred with the SCFA mix as well, suggesting SCFA as main drivers of the modulations induced by the MTB mix on immune markers, as already mentioned for IEB markers. Notably, certain metabolites had opposite effects on immune marker expression. For example, SCFA decreased *CXCL8* expression, while GABA increased it, but the overall effect with the MTB mix was a reduction. The effects of SCFA on immunity are well documented, especially in the colon, where they are known for their inflammation modulation properties and role in immune tolerance.[18,19] Hence, SCFA at concentrations similar or lower than those in the colon (2.5 to 0.625 mM) reduced IL-8 gene expression and production in a Caco-2 model stimulated by tumor necrosis factor alpha.[17] In our study, under non-inflammatory conditions and at HM concentrations, SCFA also decreased *CXCL8* expression, likely due to acetate. Similarly, *MYD88* was down-regulated by SCFA, in line with *in vivo* studies showing that microbiota-derived SCFA inhibit the TLR4/MyD88/NF-kB pathway, with NF-kB being the nuclear factor-kappa B.[63] SCFA influence oxidative stress responses differently depending on the model,[64,65] and in the present study, they reduced *GPX2* expression. SCFA influence many regulators and pathways,

such as NF-kB, extracellular signal-regulated kinases (ERK), p38 mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinases (JNK), and tyrosine-protein kinase (SYK), which explains their impact on immune function.[17] Furthermore, as mentioned for the effect on IEB, the effect of the MTB mix is not just a sum of individual metabolite effects. For instance, the increased expression of *AHR*, a transcription factor involved in the immune response, with butyrate, GABA, kynurenine and ILA, was not observed with the MTB mix. As a central regulator, *AHR* is tightly regulated, with several ligands including kynurenine, ILA and butyrate,[66–68] able to modulate its activity as well as its expression through feedback loop.[69,70]

Finally, the MTB mix reduced the expression of nutrient transporters and digestive enzymes, suggesting it may affect nutrient absorption. Specifically, the expression of *SLC2A1* (encoding GLUT1) and *SLC15A1* (encoding PEPT1) decreased. Lower *SLC2A1* expression with the MTB mix was also observed with SCFA, GABA, lactate, and 5-HT, possibly due to alternative energy sources being available when glucose is low.[15,71] In agreement, a RNA-seq study reported that SCFA treatment altered glucose metabolism, increasing GLUT4 and decreasing GLUT2 and GLUT9,[72] albeit at higher concentrations than the ones we used. The expression of *SLC15A1* decreased with SCFA mix while it increased with PA mix and putrescine, as already demonstrated *in vivo* after spermine supplementation.[73,74] It was also increased with GABA, although GABA is not transported by the latter.[75] So, once again, the effect of SCFA was predominant. Nevertheless, this modulation contrasts with the results of a study showing the activation of *SLC15A1* expression by 5 mM butyrate after 24 h of exposure in a Caco-2-BBE model.[76] This discrepancy may be due to the different models and the Caco-2 clone used. Finally, expression of *LCT*, encoding lactase, was decreased with the MTB mix, as it was with SCFA. In a Caco-2 study, *LCT* expression decreased after 11 days of differentiation, making it an early marker of maturation.[77] This decrease might be related to increased *ALPI*, a maturation marker that also increases after exposure to SCFA. SCFA have been shown to positively affect the differentiation and maturation of intestinal epithelium models,[78] which is consistent with our results.

The MTB mix up-regulated expression of *CHGA*, a marker of endocrine cells, suggesting an effect of the mixture on the NCI-H716 enteroendocrine cells. However, the modulation observed with SCFA, *i.e.* the reduction of *GCG* expression, was not observed with the MTB mix. This decrease, although not in line with the consensus that SCFA induce *GCG* expression, confirms some studies that have highlighted the particular behavior of NCI-H716 after exposure to butyrate.[79,80]

One of the main objectives of the study was to decipher the role of several HM metabolites on expression of genes related to various functions of the intestinal epithelium despite their low or very low concentrations in HM, in any case lower than in the gut. Here, we have clearly shown that HM metabolites, sometimes alone, but more often in mixture, have an impact on the expression of genes related to all the gut functions we tested *in vitro*. Within the MTB mix, the impact of SCFA appeared predominant, as most genes affected by the MTB mix were similarly affected by SCFA. Within the SCFA, it was mainly butyrate that drove the modulations, maybe as it was used at a concentration 3-fold higher than acetate and propionate, as found in HM. Nevertheless, the use of mixtures, by classes or with all the metabolites clearly highlighted some cumulative effects of individual metabolites, resulting in amplified or, on the contrary, attenuated effects. This was clearly demonstrated with GABA, which individually had a significant effect on the expression of genes related to all the functions screened, but whose effects in a mixture seemed to be barely visible. In line with this, we could speculate that, depending on the relative concentrations of the different metabolites in HM, different effects would be observed on the intestinal epithelium. Our study was designed based on mean and median concentrations found in HM, but large variability in the concentration of HM metabolites between individuals and studies has indeed been reported.[7–11,45] The question then arises as to the impact of these different metabolite profiles on the expression of genes related to the different intestinal functions, with possible consequences for infant health. When considering the possible consequences of different profiles of HM metabolites on neonatal health, it is important to note that although our multicellular model attempts to introduce the cellular diversity present in the intestinal epithelium, it has some limitations. In fact, it is an adult model, using cancer cell lines, but these cell lines do express genes present in infant gut such as that encoding for the enzyme lactase.[77] Another limitation of the present study is that we evaluated the effect of HM metabolites on several gut functions mainly through gene expression, which allowed an overview of the HM metabolite effects, and revealed a pleiotropic effect of HM metabolites, but this should be further corroborated by physiological data. While some of them were included in the present study such as the TEER and paracellular permeability with Lucifer Yellow, additional physiological readouts could be monitored, including the production of cytokines. Besides, interferences could occur between the HM metabolites and the intestinal microbiota metabolites, which were not considered in the present study. However, in the proximal parts of the infant intestine, the microbiota is less abundant and so are its metabolites. It is therefore possible that the metabolites of interest, despite their low concentration in the HM, act and/or are absorbed in the small intestine. We can therefore expect direct effects on the proximal part of the intestine, as investigated *in vitro* in this study, but we cannot exclude more indirect effects on the distal parts of the intestine and on the intestinal microbiota. For this reason, it would be important to explore the HM metabolite effects in *in vivo* preclinical models, in order to study them in more physiological models considering the influence of the microbiota, and the dynamic of growth and development during the early postnatal/lactation period, such as in some studies carried out on butyrate and PA.[19,34] Further studies are also needed to understand the potential matrix effects of HM on intestinal epithelium and host responses. Indeed, we studied the metabolites in a mix as

in HM, but they were added to the cellular model in a cell culture medium, which does not reflect the complexity of the HM composition and does not consider possible interference with the matrix.

Conclusions

In conclusion, our study showed that HM metabolites enhanced IEB *in vitro* and were able to modulate expression of genes involved in immune, digestive and endocrine functions. Cumulative as well as opposite effects of the different classes of metabolites were revealed, with a dominant effect of SCFA. The effects observed with the MTB mix did not correspond to the sum of the individual metabolite effects, highlighting the importance of considering the diversity of the HM metabolome on its impact on newborn physiology and development. Further studies in preclinical models mimicking better the complexity of gut physiology are warranted to better characterize the role of HM metabolites. This would open new avenues for the development of more biomimetic infant formulas.

Author contributions

I. L. H. L. Sa. B., So. B., S. E. and Y. L. L. conceived the project that led to the submission of this work. Sa. B., So. B. and S. E. designed the experiments; Sa. B., M. B. and V. R. performed experiments and acquired data and Sa. B. analyzed data. Sa. B., So. B. and S. E. interpreted results of experiments; Sa. B. prepared figures; Sa. B., So. B. and S. E. drafted manuscript; all authors approved final version of manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Data availability

All data generated or analysed during this study are included in this published article and its ESI† (**https://doi.org/10.57745/8Z0LWX**).

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