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Acute effects of milk polar lipids on intestinal tight junction expression: towards an impact of sphingomyelin through the regulation of IL-8 secretion?☆

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Abstract

Milk polar lipids (MPL) are specifically rich in milk sphingomyelin (MSM) which represents 24% of MPL. Beneficial effects of MPL or MSM have been reported on lipid metabolism, but information on gut physiology is scarce. Here we assessed whether MPL and MSM can impact tight junction expression. Human epithelial intestinal Caco-2/TC7 cells were incubated with mixed lipid micelles devoid of MSM (Control) or with 0.2 or 0.4 mM of MSM *via* pure MSM or *via* total MPL. C57Bl/6 mice received 5 or 10 mg of MSM *via* MSM or *via* MPL (oral gavage); small intestinal segments were collected after 4 h. Impacts on tight junction and cytokine expressions were assessed by qPCR; IL-8 and IL-8 murine homologs (*Cxcl1*, *Cxcl2*) were analyzed. *In vitro*, MSM increased tight junction expression (Occludin, ZO-1) *vs* Control, unlike MPL. However, no differences were observed in permeability assays (FITC-dextran, Lucifer yellow). MSM increased the secretion and gene expression of IL-8 but not of other inflammatory cytokines. Moreover, cell incubation with IL-8 induced an overexpression of tight junction proteins. In mice, mRNA level of *Cxcl1* and *Cxcl2* in the ileum were increased after gavage with MSM *vs* NaCl but not with MPL. Altogether, these results suggest a specific action of MSM on intestinal tight junction expression, possibly mediated by IL-8. Our study provides clues to shed light on the beneficial effects of MPL on intestinal functions and supports the need for further mechanistic exploration of the direct *vs* indirect effects of MSM and IL-8 on the gut barrier.
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1. Introduction

Milk and dairy products are important nutritious foods worldwide. The International Dairy Federation estimated the average consumption of dairy products in the world to 111 kg in 2016 per year and per person. Bovine milk contains 3.5–5% of lipids and fat is present in milk as milk fat globules, comprising a core of triglycerides (TG, ~98%) surrounded by their milk fat globule membrane (MFGM). MFGM is a tri-layered biological membrane rich in bioactive phospholipids/polar lipids (PL) (~0.5–1%) [1]. Dairy products are thus a natural source of PL, which have interesting natural functional properties for the food industry as emulsifiers/texture promoters [2] and may exert beneficial effects on health [3–5]. Interest has also recently grown on using milk polar lipids (MPL) as potential nutraceutical, notably *via* rich sources such as buttermilk [3]. Indeed, several rodent studies have demonstrated that MPL are able to decrease hepatic lipid accumulation [6] and intestinal cholesterol absorption [7]. MPL have also beneficial properties to modulate lipid absorption and postprandial lipemia [8] and to inhibit the growth of cancer cells [9]. MPL have a specific PL profile with a large proportion of SM (~24%), in comparison with other sources of PL products. Indeed, soybean PL are devoid of SM and egg PL contain ~1.5% of SM [10]. A recent study in mice reported that milk-SM (MSM) had a beneficial impact on hepatic lipid accumulation, decreased muscle inflammation and lowered pro-inflammatory endotoxemia [11], which is one reported consequence of altered gut barrier [12,13]. Moreover in comparison with soybean PL (major vegetal source of PL), MPL supplementation can induce (i) lower expression of markers of inflammation and of macrophage infiltration in epididymal adipose tissue and (ii) more goblet cells in colon [14]. Furthermore, in rat pups MPL [15] and MSM [16] are implicated in intestinal development. For example, villi, crypts and Paneth cells are similar after MPL-based milk formula and after mother milk, while they are altered after vegetable fat-based milk formula [15]. Altogether, these results suggest various effects of MPL on the

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intestine, a major function of which is the intestinal barrier. One major actor of the gut barrier is tight junction proteins, which represent a physical barrier to protect the host against potentially dangerous elements. Tight junctions consist of transmembrane proteins such as occludin, claudin and the junctional adhesion molecule 1 (JAM-1) interacting with intracellular proteins, as zonula occludens (ZO) themselves connected to the cell cytoskeleton proteins (actin filaments) [13]. Nutrients including proteins, additives and lipids have been shown to modify the tight junction proteins and thereby impact intestinal paracellular permeability [17]. Indeed, lauric acid and long-chain fatty acids (FA) such as palmitic acid, oleic acid, EPA and DHA increased paracellular permeability [17,18] and animals fed a high fat diet have decreased expression of intestinal tight junction proteins claudin-1 and -3, JAM-1 and Occludin [19]. Furthermore, it was clearly demonstrated that obesity [20] and metabolic diseases [21] are linked to increased intestinal permeability and translocation of bacteria or bacterial products like endotoxins from the intestine to the liver and to other tissues. However, in the frame of metabolic diseases, the possible links between MSM and an increased expression of tight junction proteins were only scarcely studied up to date and underlying mechanisms remain largely unknown.

Dietary metabolites in the digestive tract can modify gut barrier, but other types of bioactive compounds can also impact the intestine, which in turn could also act as indirect mediators for the action of nutrients on the intestine. Interleukins were described as implicated in tight junction regulation in Caco-2 cells [22–25]. Notably interleukin-8 (IL-8), a member of chemokine superfamily, stimulates the migration of cells including neutrophils, monocytes, lymphocytes, and fibroblasts into inflamed tissues *in vivo* [26–29]. Furthermore, IL-8 also stimulates colonic epithelial cell migration in vitro [30]. In humans, IL-8 was also proposed as a major actor responsible for the development of the intestine [31] and could have impacts on gut barrier. In mice, keratinocyte chemoattractant (KC) (CXCL1) and macrophage inflammatory protein-2α (Mip-2) (CXCL2) are suggested to be functional homologs of IL-8 [32,33].

The aim of our study was to characterize the impact of MPL and MSM on the gene expression of tight junction proteins in vitro in Caco-2/TC7 cells and to perform a mechanistic exploration of the potential direct impact of IL-8. The effects of MPL and MSM on intestinal barrier and on the murine IL-8 homologs were also studied *in vivo* by oral gavage in mice.

2. Methods

2.1. MPL preparation

MSM (bovine) and egg-sphingomyelin (ESM, chicken) were purchased at >99% purity from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). C16-Ceramide (C16-Cer, d18:1/16:0) was purchased at 98% purity from Sigma (Saint-Quentin-Fallavier, France). The MPL-rich ingredient was a phospholipid concentrate from butterserum (most MPL-rich type of buttermilk obtained from anhydrous milkfat production), kindly provided by Corman (Limbourg, Belgium). It was purified using Folch extraction followed by two subsequent acetone extractions. The extract of MPL-rich ingredient contained >78% of PL, the balance consisting of TG. The detailed PL profile is available in Table 1 and is consistent with the literature [10]. The SM profile of MSM, ESM and MPL-rich ingredient (Fig. S1) was analyzed by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) as previously described [34]. As expected, the FA species of ESM had less diversity than in MSM and were mostly composed of C16; in turn the FA profile of commercial pure MSM and of SM within MPL were similar.

2.2. In vitro

2.2.1. Culture preparation

Caco-2/TC7 intestinal cells, provided by Dr. Rousset (INSERM U505, Paris, France), are derived from a human adenocarcinoma. They are able to undergo differentiation into polarized epithelial cells that show a brush border phenotype and form well-developed and functional tight junction complexes [35]. The TC7 clone, compared to the parental cell line, has increased expression of markers associated with differentiation of enterocytes [36]. Cells were routinely cultured in high-glucose (4.5 g/L) medium with glutamine (DMEM GlutaMAX; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% fetal calf serum (v/v), 1% of nonessential amino acids (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and 1% antibiotics (penicillin/streptomycin; Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), and maintained under a 10% CO_2 atmosphere at 37°C. Cells were seeded at a density of 2.5×10^5 cells per Transwell filter inserts (12 mm polycarbonate, 0.4 μm pore size Costar, Cambridge, MA, USA) in 6-well plates. The experiments were performed after 21 days of culture on semi-permeable filters. Prior to lipid treatment, cells were incubated with serum-free complete medium for 24 h. The incubation media containing so-called "mixed micelles", mimicking postprandial lipids in the gut during digestion [37], were prepared as follows: oleic acid (0.5 mM), 2-monooleylglycerol (0.2 mM), cholesterol (0.05 mM), taurocholic acid (2 mM), L-α-lysophosphatidylcholine (0.2 mM), phosphatidylcholine (0.4 mM). For the preparation of lipid micelles, stock solutions were prepared in chloroform-methanol. Appropriate volumes were added in glass tube, dried under a stream of nitrogen. Lipid mixtures were frozen at −20°C until the experiment. The day of the experiment, lipids were vortexed in serum-free complete medium and further dispersed in an ultrasonic bath for 30 min at 37°C. Cells were incubated during 24 h with micelle-containing medium into the apical media and the basolateral compartment received serum-free complete medium complemented with 1% nonessential amino acids, 1% pyruvate and 1% antibiotics.

Different experiments were conducted in which: (i) MPL-rich ingredient or MSM were added at the corresponding concentration of 0.2 or 0.4 mM of SM into the micelles in culture medium (i.e., considering average molecular weight of pure MSM: 0.16 or 0.31 mg/mL). PC was added to equilibrate micelles at 0.4 mM. Analyses were performed on three independent experiments; (ii) MSM was added into the micelles at the corresponding concentration of 0.2, 0.4 and 0.6 mM in culture medium. mRNA levels were performed on three independent experiments and IL-8 secretion were performed on five independent experiments. For 0.6 mM condition, only two independent experiments were performed. All experiments were realized in triplicate. Of note, our rationale for the tested SM proportions within lipids incubated onto Caco-2 cells was elaborated in a nutraceutical approach. Our previous analyses showed that SM content in MPL-rich products, buttermilk and butterserum, was 5–13% of total lipids [34]. Moreover, it has been reported that 20% of MSM would remain intact during human digestion [38] while only <1% of FA from other lipids (TAG, PL) remain unabsorbed [39]. Therefore, we calculated the expected molar ratio of intact SM to free FA to be in the order of 1:3 from buttermilk to 1:1 from butterserum. The amount of SM that we incorporated in model micelles corresponds to this range of SM:FA ratios.

To evaluate if a digestion byproduct of MSM can induce similar effects like intact MSM, we performed similar incubations with micelles enriched with 0.2 or 0.4 mM of C16-Cer, the major form of ceramide derived from MSM found in human ileostomy [38]. Cells were incubated during 24 h and mRNA levels were performed on one independent experiment in triplicate. To evaluate if the impact of

Table 1
Polar lipid profile of the MPL-rich ingredient used for *in vitro* and in vivo studies [1]

PL classes, % total PL	SM	PC	PE	PS	PI
MPL-rich ingredient	25.9± 0.2	27.3±1.5	24.8±0.1	11.9±0.9	9.8±0.4

Each value represents the mean±S.E.M. of the values for $n=2$ batches of extracted MPL.
[1] MPL, milk polar lipids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, polar lipid; (here glycerophospholipids) PS, phosphatidylserine; SM, sphingomyelin.

MSM is due to the specific FA and sphingoid base composition of milk, we also evaluated the impact of ESM on tight junction. Mixed micelles were supplemented with 0.2 or 0.4 mM of MSM or ESM in culture medium (*i.e.* considering average molecular weight of pure ESM: 0.14 or 0.29 mg/mL). Cells were incubated during 24 h and mRNA levels were performed on three independent experiments.

We verified that the hydrodynamic diameter of incubated lipid structures was not dramatically altered by differences in lipid composition using Dynamic Light Scattering (Zetasizer NanoS, Malvern, UK). "Mixed lipid micelles" media contained both structures of micellar size (within the range 8–40 nm) and of lipid vesicle size (in the range 150–300 nm). Importantly, this is altogether consistent with the size of lipid structures reported in duodenal lipid digestion content in humans [37,40] and was similar regardless of micelle composition. The size of micelles *stricto sensu* was similar regardless of inclusion of milk or egg SM.

Another experiment was conducted: recombinant human IL-8 was incubated during 24 h at 100 pg/mL on apical media and/or 80 pg/mL on basolateral media. These concentrations were used to mimic the previously observed concentrations in apical and basolateral media after MSM incubation. mRNA levels were analyzed on one experiment performed in triplicate. Recombinant human Interleukin-8 human (rh IL-8) was obtained at >98% purity from Sigma (Saint-Quentin-Fallavier, France).

The integrity of cell monolayer was checked by measuring the transepithelial electrical resistance (TEER). TEER values were recorded in culture medium at 37°C with chop-stick electrodes. After treatment, cells and media were collected and frozen at −80°C for further analysis.

2.2.2. Measurement of paracellular permeability

Cellular permeability was evaluated for cells incubated with total MPL or pure MSM by measuring the emitted fluorescence at 530 nm for FITC-Dextran (excitation at 485 nm) and at 520 nm for Lucifer Yellow (excitation at 490 nm). In brief, 1 mg/mL of FITC/dextran (Sigma, Saint-Quentin-Fallavier, France) or 45.7 μg/mL of Lucifer Yellow (4 kDa, Sigma, Saint-Quentin-Fallavier, France) were added in the apical side of the transwell chamber. Medium from the basolateral side was collected, and fluorescence intensity was measured using a fluorescence spectrophotometer (SAFAS Xenius XL, SAFAS Monaco, France) after 120 min.

2.2.3. Immunofluorescence

After two washes with PBS, monolayers were fixed in 100% methanol at −20°C and permeabilized with TRITON × 100. Cells on filters were then rinsed in PBS and incubated with anti-mouse occludin (dilution 1:100, Santa Cruz Biotechnology, CA, USA) during 1 h at room temperature. After PBS washes, filters were incubated with goat anti-rabbit IgG conjugated to Alexa488 (dilution 1:1000, Life technologies SAS, Saint-Aubin, France). ProLong glass antifade mountant with NucBlue (Hoechst) stain was used as a nuclear counterstain (Thermo Fisher Scientific, Waltham, MA, USA). Images were obtained under Axiovert 200 M microscope (Carl Zeiss, Göttingen, Germany) by AxioVision software.

2.2.4. Quantification of cytokine secretion

Concentrations of cytokines into the apical and basolateral compartments were performed by ELISA kits according to the manufacturer's instruction for IL-8 (Life Technologies SAS, Saint-Aubin, France), Tumor necrosis factor-alpha (TNF-α) (MyBioSource, San Diego, CA, USA), IL-6, IL-17, IL-22 and Monocyte Chemoattractant Protein-1 (MCP-1) (Abcam, Cambridge, United Kingdom).

2.3. In vivo

2.3.1. Animals and experimental protocol

C57BL/6 mice (male, 19.4±0.2 g, 8-weeks-old; Janvier SA, Saint Berthevin, France) were housed 5 per cage in a temperature-controlled room (24±1°C, 12 h daylight cycle, free access to food and water). After two weeks of acclimatization with a chow diet, they were randomly divided into groups of 10 mice (5 mice/cage). On the experimental day, after overnight fasting, mice received by oral gavage, diluted in NaCl:

- MSM 1: 200 μL of 25 mg/mL of MSM (corresponding to 5 mg)
- MSM 2: 200 μL of 50 mg/mL of MSM (corresponding to 10 mg)
- MPL 1: 200 μL of 96.2 mg/mL of MPL (corresponding to 5 mg of SM)
- MPL 2: 200 μL of 192.3 mg/mL of MPL (corresponding to 10 mg of SM).

Of note, using the Human Equivalent Dose calculation of the FDA [41], 5 mg and 10 mg of SM in mice would correspond to a bolus of 0.8 g and 1.6 g for a human of 60 kg body weight, respectively, *i.e.*, the amount within an intake of 0.3 to 0.6 L of butterserum.

A group received saline only and was used as control. Mice were euthanized 4 h after gavage. For euthanizing, mice were anesthetized using isoflurane (4% isoflurane in 100% O_2 until unconscious and anesthesia was maintained with 2% isoflurane in 100% O_2). After laparotomy, blood was collected by portal vein with heparinized syringes and mice were killed after a terminal blood collection by cardiac puncture. Intestines were collected after a wash of the lumen with PBS. Whole sections of intestine were immediately frozen in liquid nitrogen and stored at −80°C before analyzed by RT-qPCR. All procedures were approved by the Animal Ethic Committee of University Lyon 1.

2.3.2. Plasma biochemical analysis

Plasma samples from cardiac puncture and portal vein were obtained after centrifugation (3600×*g*, 15 min, 4°C). Plasma concentrations of IL-6, TNF-α, IL-1β, IL-10, MCP-1, CXCL1 (limit of detection: 6 pg/mL) and CXCL2 (limit of detection: 160 pg/mL) were determined using a multiplex immunoassay kit (Milliplex® MAP Mouse Cytokine, Mcytomag-70 K, Merck Millipore) and measured using a Bioplex 200 system (Biorad, CA, USA).

2.4. RNA extraction and quantitative real-time PCR

Total RNA was extracted from whole intestine segments of mice and Caco-2/TC7 cells with TRI Reageant (Sigma, Saint-Quentin-Fallavier, France) and suspended in RNase-free water. RNA concentration was measured with Multiskan GO microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and RNA samples with A260/280 ratio between 1.7 and 2.1 were considered of good purity. Reverse transcription (RT) was performed using PrimeScript RT reagent kit (Ozyme, Saint Quentin en Yvellines, France) with one microgram of RNA. Real-time PCR assays were performed using a Rotor-Gene Q (Qiagen, Hilden, Germany) and SYBR qPCR Premix Ex Taq (Tli RNaseH Plus) reagents. PCR primers are listed in Supplemental Table 1. The results were normalized by using TBP (TATA box binding protein) mRNA concentration, measured as reference gene in each sample.

2.5. Statistical analysis

All data, presented as means±S.E.M., were analyzed with Graph Pad Prism Software (version 6.01, San Diego, CA, USA). Normality of data was examined using Shapiro–Wilk test. One-way analyses of variance (ANOVA) were performed on normal data. For non-normal data or small number of observations, a Kruskal-Wallis test was performed followed by a Dunn post hoc test. Two-way ANOVA were used to assess whether there were significant differences between source of SM (pure or in total MPL) within dose (values bearing different superscript letters were significantly different). When the interaction term was not significant this analysis was followed by Bonferroni post hoc multiple comparisons test. If the interaction term was significant an unpaired test was performed to determine differences between MSM and MPL groups. One-way ANOVA

followed by a post hoc Dunnett test were performed to assess statistical differences from control (mixed micelles without SM or NaCl group in the *in vivo* study) (an asterisk represents a significant difference with control).

3. Results

3.1. Pure sphingomyelin in model postprandial lipid micelles increases the gene expression of tight junction proteins and induces IL-8 secretion in Caco-2/TC7 cells

Because MPL contain ~24% of MSM, we investigated the impact of pure MSM *vs* total MPL on Caco-2/TC7. Increasing concentration of MSM or MPL did not affect Caco-2 TEER after 24 h incubation (data not shown). As shown in Fig. 1, the SM source (pure MSM or MPL including MSM at the same dose) had a significant impact on the gene expression of a transmembrane protein of tight junctions, the JAM-1 (Fig. 1B, P_{source} < .05). The gene expression of Occludin, another transmembrane protein, and ZO-1, an intracellular protein, were significantly higher for 0.4 mM of MSM compared to other groups, and these effects were specific of pure MSM (Fig. 1A, C). In comparison with control mixed micelles devoid of MSM, we observed a significant increase of mRNA level of Occludin (P<.05 for 0.2 mM of MSM and P<.0001 for 0.4 mM of MSM) and ZO-1 (P<.05 for 0.2 mM of MSM and P<.0001 for 0.4 mM of MSM) (Fig. 1A, C). No difference was observed when cells were incubated with mixed micelles supplemented with total MPL. Compared with control mixed micelles devoid of SM, pure MSM increased significantly the gene expression and secretion of IL-8 (P<.05 for 0.2 mM of MSM and P<.01 for 0.4 mM of MSM) while total MPL did not (Fig. 1D, E), despite containing the same SM amount. IL-8 secretion increased in the apical compartment with pure MSM (P<.05 for 0.4 mM of MSM) and also in the basolateral compartment (P<.05 with 0.2 mM and P<.05 with 0.4 mM of pure MSM in comparison with mixed micelles devoid of SM). Total MPL incorporated in mixed micelles did not induce a significant release of IL-8 (Fig. 1D and E), confirmed by a source dependent effect for IL-8 secretion (Fig. 1D, P_{source}<.01) and for its mRNA expression (Fig. 1E, P_{source}<.01). Of note, gene expression of another inflammatory marker, MCP-1, was not impacted by MSM (results not shown). Moreover, isolipidic lipid micelles containing increasing proportions of MSM confirmed that the impact of MSM on tight junction proteins and IL-8 observed above was due to increased amount of MSM and not to the higher total lipid content of the micelles following addition of MSM (data not shown). A major digestion product of MSM, namely C16-Cer, did not induce all the above-mentioned impacts on Caco-2/TC7 cells (Fig. S2). Of note, effects of SM were not specific of milk origin. Indeed, we also observed highest gene expression of tight junction proteins and an increase of IL-8 secretion and expression with

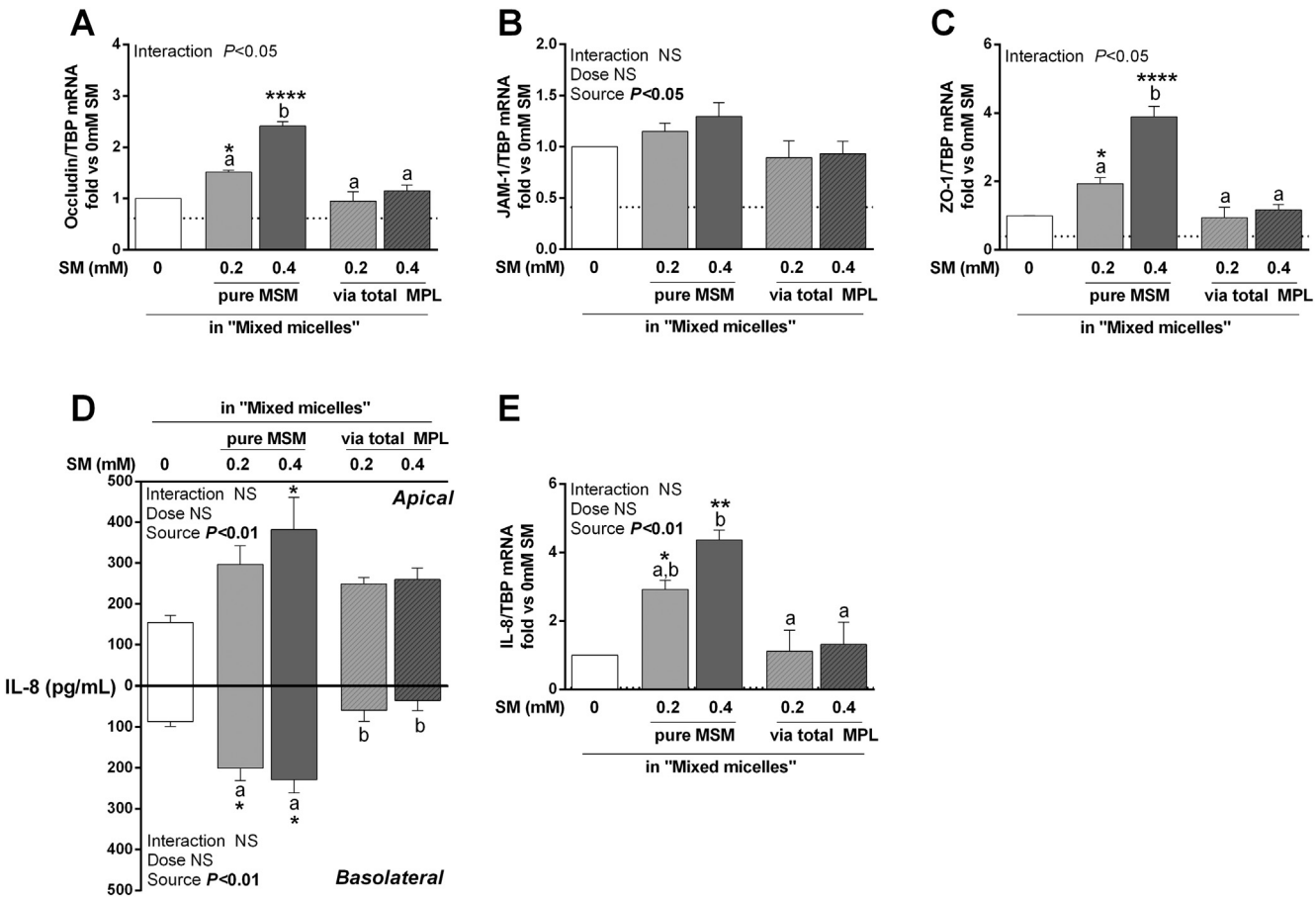

Fig. 1. Milk sphingomyelin specifically increased tight junction protein mRNA and stimulated the expression and secretion of IL-8 in Caco-2/TC7 cells. mRNA was measured by qPCR for (A) occludin, (B) JAM-1, (C) ZO-1, and (E) IL-8. (D) Concentration of IL-8 in the apical and basolateral compartment was evaluated. Values represented the mean±S.E.M. (Three independent experiments in triplicate). Data were analyzed by two-way ANOVA (values bearing different superscript letters were significantly different) and the mean of each condition were compared with mixed micelles devoid of MSM (*P<.05 using post hoc *vs* 0 mM). Gene expression values were normalized to TBP mRNA. Dotted lines represent the mean value of a DMEM condition. Of note, 0.2 mM of MSM corresponds to 0.16 mg/mL. IL-8, Interleukin-8; JAM-1, Junctional adhesion molecule 1; MPL, milk polar lipids; MSM, Milk-sphingomyelin, TBP, TATA-binding protein; Zo-1, Zonula occludens-1.

ESM (Fig. S3), which differs from MSM by the FA (Fig. S1) and sphingoid base composition [42].

3.2. Sphingomyelin induces a dose-dependent secretion of IL-8 that can be a possible effector of the impact on the gene expression of tight junction proteins

We then more specifically studied the effects of MSM on paracellular permeability and tight junction proteins. MSM caused no modification in Caco-2/TC7 TEER after 24 h (data not shown). Furthermore, there were no differences among treatments on cell permeability to FITC-conjugated dextran or Lucifer Yellow (Fig. S4A, B). Caco-2/TC7 monolayers exhibited uniform fluorescent nuclear staining for the treatments, confirming the lack of toxicity and the tight junction integrity. The protein expression of occludin seemed to be higher for the treatment with 0.4 mM MSM *vs* control (Fig. S4C).

A complementary study was performed to test the dose–response impact of MSM on Caco-2/TC7 cells from 0.2 to 0.6 mM. Of note, control micelles devoid of MSM did not have a different impact on the expression of tight junction proteins than DMEM culture medium devoid of micelles (dotted line). There was a significant increase in gene expression of Occludin with 0.4 mM of MSM (Fig. 2A, $P<.05$). For JAM-1, incubation with micelles containing 0.4 mM of MSM led to an increase of mRNA expression (Fig. 2B, $P<.05$). The gene expression of ZO-1 was also significantly higher for MSM 0.4 mM ($P<.01$) and MSM 0.6 mM ($P<.05$) compared to control (mixed micelles devoid MSM) (Fig. 2C). The mRNA level of Claudin-1 was no different between treatments (Fig. 2D). Both in the apical and basolateral compartments, dose–response secretions of IL-8 were observed after apical stimulation with the micelles containing MSM (Fig. 2E, also showing Spearman correlations between MSM concentration and IL-8 secretion). The 0.6 mM SM treatment induced a significant increase of IL-8 secretion in both compartments, compared to control lipid micelles. ($P<.05$). Gene expression of IL-8 increased significantly for 0.4 mM MSM ($P<.05$ *vs* control, Fig. 2F). As shown in Fig. 2G, there was a positive correlation between apical and basolateral IL-8 concentrations ($r=0.94$, $P<.0001$) suggesting a common mechanism. This SM-induced secretion was specific of IL-8 as we observed no impact on IL-6, IL-17, IL-22, TNF-α and MCP-1 secretions (data not shown) and no differences on the gene expression of IL-1β and MCP-1 (Fig. S4D, E).

To evaluate whether MSM impact could in fact be mediated by the increased IL-8 secretion, we tested the impact of recombinant human IL-8 on the gene expression of tight junctions. After a 24 h challenge with recombinant human IL-8 on apical and/or basolateral media on Caco-2/TC7 cells, TEER was unaffected (results not shown). However, mRNA expression of the tight junction proteins Occludin, JAM-1, and ZO-1 was upregulated compared to the untreated cells (DMEM) (Fig. 3A–C). We also observed an upregulation of mRNA level of IL-8 (Fig. 3D).

3.3. Milk sphingomyelin and total milk phospholipids can both increase the gene expression of IL-8 homologs in ileum in vivo in mice

We finally performed a study in mice orally force-fed with MPL or MSM to evaluate their impacts *in vivo*. We specifically examined the expression of murine IL-8 homologs, *Cxcl1* (KC) and *Cxcl2* (Mip-2), in the jejunum and ileum of mice. The SM source (pure MSM or MPL including MSM at the same dose) had a significant impact on the gene expression of *Cxcl1* in the ileum (Fig. 4A, $P_{source}<.05$). *Cxcl1* and *Cxcl2* mRNA expressions were upregulated in the MSM1 mice ileum compared with mice forced-fed with NaCl (Fig. 4A, B, $P<.01$). We also observed an overexpression of *Cxcl2* for MPL1 mice in comparison with NaCl mice (Fig. 4B, $P<.05$). However, no significant impact was observed in jejunum for *Cxcl1* (KC) and *Cxcl2* (Mip-2) (Fig. S5). The concentration of IL-8 homologs showed no differences among groups in the portal vein (directly derived from the intestine) nor systemic circulation (Fig. 4C–F). Of note, IL-1β, TNF-α, IL-6, IL-10 and MCP-1 secretions were indistinguishable in portal vein and systemic circulation (data not shown). Altogether, the outcome most impacted by MPL and MSM was *Cxcl2* expression in the ileum. Moderate effects on the expression of tight junction proteins were observed (Fig. S6). Indeed, the SM source had a significant impact on the gene expression of *Occludin* (Fig. S6A, B, $P_{source}<.05$ in the duodenum and jejunum) and *Zo-1* (Fig. S6G, I, $P_{source}<.05$ in the duodenum and ileum).

4. Discussion

The gastrointestinal tract is a complex interface between the external environment and the immune system, establishing a dynamic barrier that enables the absorption of dietary nutrients and the exclusion of potentially harmful compounds from the intestinal lumen. The expression of tight junction proteins, implicated in intestinal permeability and barrier function [43], can be regulated by dietary components [17]. Tight junctions are not static barriers but highly dynamic structures that are constantly being remodeled due to interactions with external stimuli, such as food residues and pathogenic and commensal bacteria. Up to date, different studies examined the impact of the amount and FA profile of dietary fat on tight junctions [18,19]. Study showed that gangliosides, which are complex sphingolipids, can protect tight junctions after an endotoxin challenge [44]. Short chain FA, especially butyrate could impact tight junction permeability [45] and assembly of tight junction [46]. In this context, the interest for such functional ingredient both is growing, on a scientific point of view and for the food industry. Dietary sphingolipids of milk origin, which are present in the native complex MPL, could present benefits regarding the prevention of lipid metabolism [6–8] and inflammation disorders [9,14,47]. Notably, published data contribute to propose MPL-rich sources such as buttermilk/butterserum as candidates for developing bioactive food ingredients or nutraceuticals [3–5,34]. However to date, little is known about the impact of MPL and also of MSM on tight junctions. Norris et al., have observed that a high fat diet enriched in MSM induced a decrease of endotoxemia in mice [11]. This protective effect could be mediated by a mechanism of gut barrier enhancement. However, authors demonstrated no impact of MSM on tight junction gene expression nor on paracellular permeability in mice, while other reported increased colonic expression of *Occludin* and *ZO-1* but no beneficial impact on gut permeability in a model of mice challenged with LPS [48].

In vivo, sphingolipids are considered to be highly bioactive compounds and their endogenous metabolism can also be modified by constituents of the diet with consequences for cell regulation and disease [49,50]. Sphingolipid composition of biological membranes in the body consists mainly of SM, as is also the case for MPL. Here we studied the links between dietary SM (pure or within MPL) and the expression of tight junctions in acute in vitro and *in vivo* studies. In Caco-2/TC7 cells, upon incubation with mixed lipid micelles containing pure MSM or total MPL, an overexpression of tight junction proteins was observed with increased concentrations of MSM but not with MPL (naturally containing equivalent amounts of MSM). Of note, we verified that the global composition of SM species in pure MSM and in MPL was similar. Therefore, it should now be elucidated whether this lack of impact of MPL on tight junction expression is due to (i) specific effects of other MPL species, including phosphatidylethanolamine, -serine and -inositol (alone or synergistically, and possibly due to their interfacial/colloidal properties), (ii) a lower bioaccessibility of MSM for intestinal cells when incorporated within MPL, or (iii) the higher total lipid concentration in MPL incubation conditions *vs* MSM micelles. In MDCK cells, enhancement of barrier function is correlated with higher expression of occludin [51].

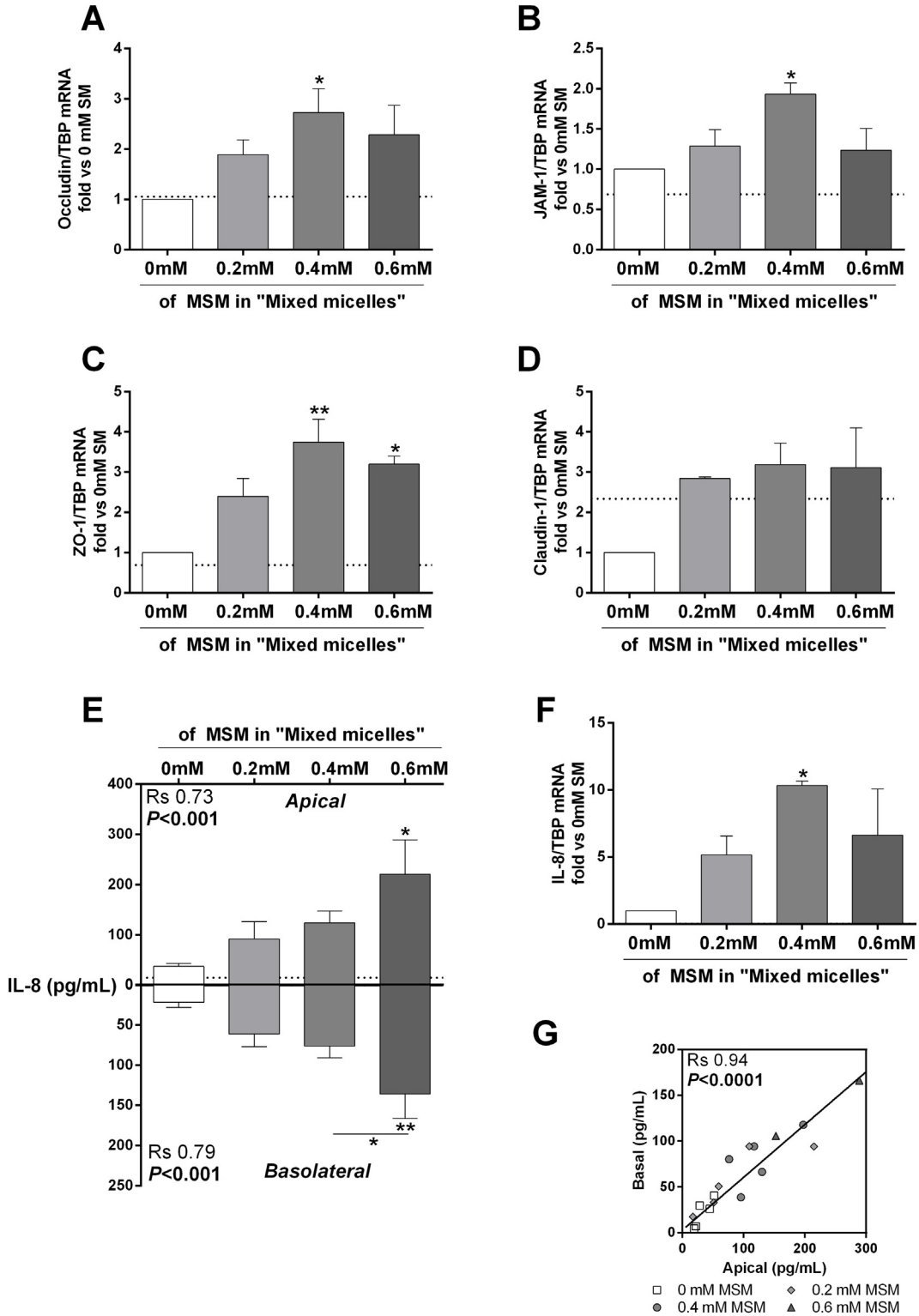

Fig. 2. Milk-sphingomyelin increased mRNA expression of tight junction proteins and polarity secretion and gene expression of IL-8. MSM induced a higher expression in Caco-2 of (A) Occludin, (B) JAM-1, (C) ZO-1 and (D) claudin-. A polarity secretion (E) and gene expression (F) of IL-8 by Caco-2/TC7 cells after treatment with mixed micelles supplemented with or without MSM were observed. (G) A positive correlation between apical and basolateral concentration was observed (correlation was analyzed by Spearman). (Three independent experiments in triplicate for 0, 0.2 and 0.4 mM and five independent experiments in triplicate for IL-8 secretion. Two independent experiments in triplicate for 0.6 mM). Gene expression values were normalized to TBP mRNA. Dotted lines represent the mean value of a DMEM condition. Data are reported as mean fold *vs* 0 mM MSM±S.E.M. Data were analyzed by one-way ANOVA (**P*<.05, ***P*<.01, ****P*<.001 and *****P*<.0001). Rs, Spearman correlation. Of note, 0.2 mM of MSM corresponds to 0.16 mg/mL. IL-8, Interleukin-8; JAM-1, Junctional adhesion molecule 1; MSM, Milk-sphingomyelin, TBP, TATA-binding protein; Zo-1, Zonula occludens-1.

Consistently, several studies have shown a correlation between a decrease in occludin expression and an increase in epithelial tight junction permeability, *i.e.* a decreased gut barrier function [52]. Therefore, the increased expression of occludin with MSM in the present study can be considered as an early signal of gut barrier enhancement, even if we did not observed any acute modification of

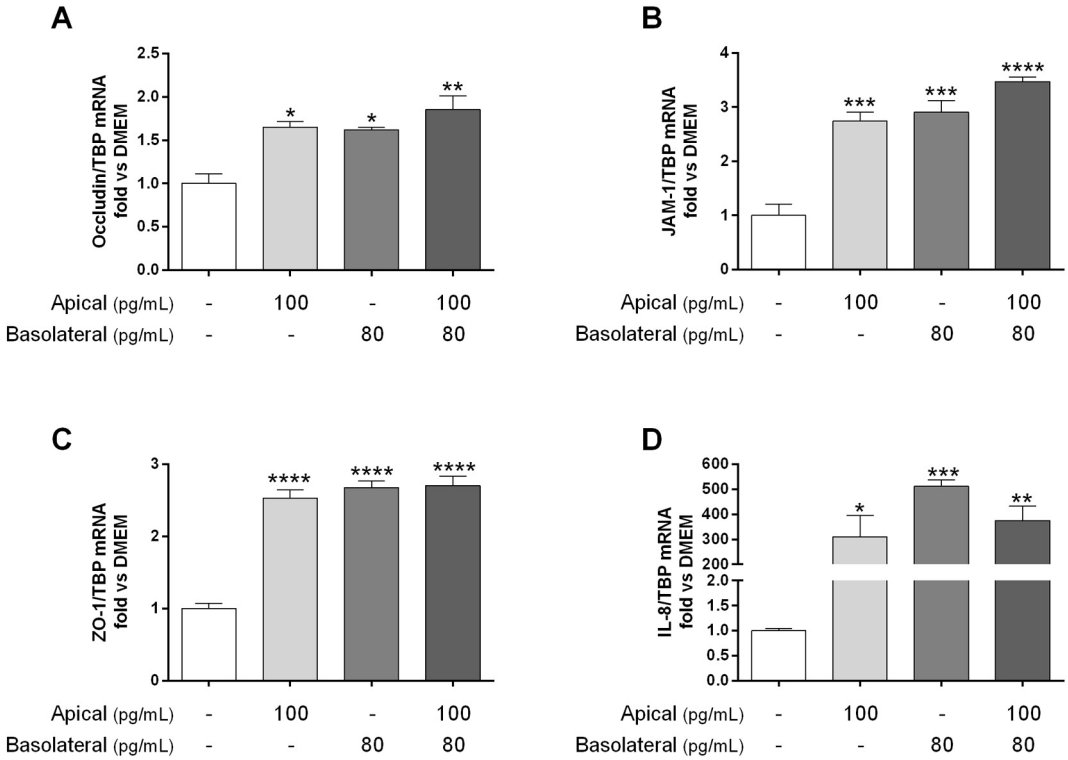

Fig. 3. IL-8 that could be a possible effector of the impact on the gene expression of tight junction proteins. Caco-2/TC7 cells were incubated with recombinant human IL-8 during 24 h. Gene expression of (A) occludin, (B) JAM-1, (C) ZO-1 (D) and IL-8 were performed. Values represented the mean±S.E.M. within one experiment (One experiment in triplicate). An asterisk represents a significant difference with DMEM condition at $P<.05$. IL-8, Interleukin-8; JAM-1, junction adhesion molecule 1; TBP, TATA-binding protein; Zo-1, Zonula occludens-1.

epithelial tight junction permeability in the present short-term conditions.

A possible molecular mechanism by which SM induced an increase in the gene expression of the tight junction proteins was then explored. Regulation of the assembly, disassembly, and maintenance of tight junction structure is influenced by various physiological and pathological stimuli. Cytokines, such as TNF-α, interferon gamma (IFNγ) [53], IL-4 and IL-13 [54] have been reported to decrease barrier function [55]. Importantly, Motouri et al. showed that MSM plays an important role in neonatal gut maturation during the suckling period [16]. Furthermore, IL-8 present in human milk has been identified as a major actor responsible for the development of the intestine. IL-8 has been shown to increase cell migration, proliferation and differentiation when Caco-2 cells were treated with rh IL-8 in vitro [31]. We therefore emitted a novel hypothesis regarding a link between dietary SM, IL-8 secretion and the intestinal barrier, which had not yet been established in the literature. In this study, micelles containing SM induced an overexpression of IL-8 and a dose–response vectorial secretion, which we propose can be involved in the observed modulation of tight junctions. It has been shown that TNF-α induces IL-8 secretion and increases tight junction permeability [23]. However, in our study, no impact of MSM on TNF-α nor MCP-1 and IL-1β were observed. *In vivo*, IL-8 is known for its involvement in the inflammatory process [29]. A basolateral secretion of IL-8 plays a role in the recruitment of circulating neutrophils from the bloodstream to the site of tissue injury or infection. Moreover, it is speculated that apically secreted IL-8 may initiate or increase the pathway responses in epithelial restitution before any potential loss of intestinal barrier integrity because of toxin exposure [56–59]. Thus considering the involvement in the development of the immature intestine of both IL-8 [31] and dietary SM [16], this could also suggest a role of both SM and IL-8 in the morphology of the mature intestine.

We therefore aimed to explore whether IL-8 could be partly responsible for the modification of tight junction expression by MSM (indirect impact of SM on tight junction *via* IL-8 secretion). We observed an overexpression of tight junction expression when cells were incubated with recombinant human IL-8, suggesting that the impact of MSM can be partly due to a specific action of IL-8. It is widely known that IL-8 is involved in inflammatory bowel disease [60] and is a target for inflammatory disease treatments [61,62]. However, in this study, (i) no other tested inflammatory markers were impacted and (ii) we observed higher gene expression of tight junction proteins, which are usually impaired in inflammatory bowel diseases. Evaluating the intracellular processes involved in the modulation of intestinal tight junctions and gut barrier by IL-8 will now be important to understand mechanisms involved in intestinal permeability disorders and to assess the consistency of novel therapeutic strategies for intestinal diseases. Notably, further studies should be performed by removing or mutating IL-8 or its receptor in the Caco-2 cells (*e.g.* siRNA transfection assays) or develop transgenic mice (*e.g.* CXCR1/2-KO) to verify if the effects of MSM would be maintained (suggesting a direct effect on tight junctions) or blunted (suggesting an IL-8 mediated effect on tight junctions only). Moreover, incubation of Caco-2/TC7 cells with recombinant human IL-8 induced an increased gene expression of IL-8. Because ZO-1 has been shown to be implicated in IL-8 signaling in breast cancer cells [63] and lung cells [64], such an indirect regulation loop of IL-8 *via* ZO-1 in Caco-2/TC7 cells would now deserve to be studied.

Our *in vivo* study shows an overexpression of IL-8 homologs in the ileum when mice were gavaged with MPL or MSM (at ~5 mg or 10 mg of SM) and moderate effects on tight junction protein expressions in the gut. Intestinal secretion of interleukins had been demonstrated in several studies. Indeed, intestinal epithelial cells could secrete IL-8 in healthy human [58] and in Caco-2 cells [56]. Other epithelial cells could have a polarized secretion of interleukins, such as IL-1, IL-6 and IL-8 [65–67]. The relative functional importance of apical *vs* basolateral secretion requires further elucidation. Moreover, the impact of MSM on the secretion of IL-8

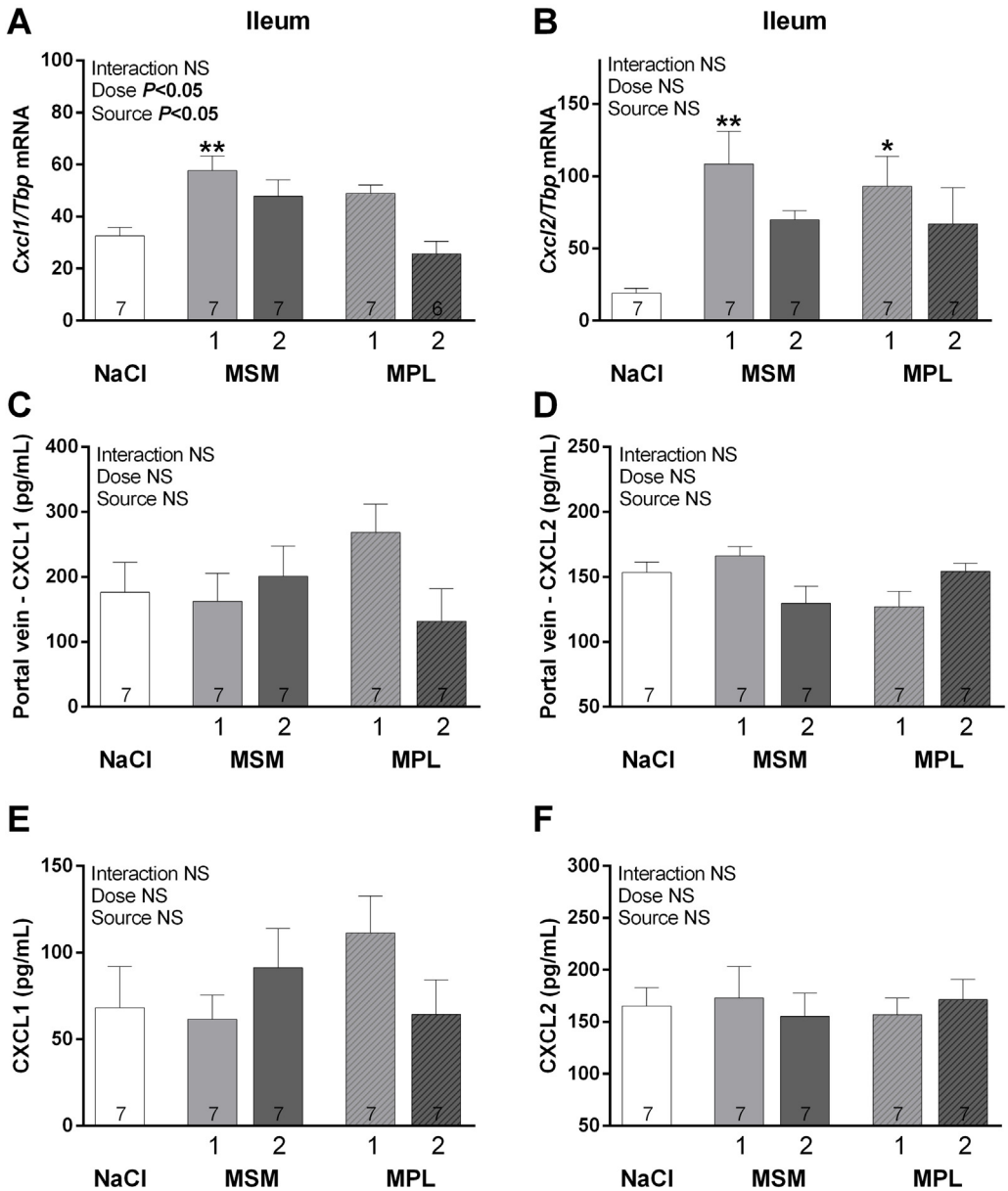

Fig. 4. Effect of milk sphingomyelin (MSM) or milk polar lipids (MPL) administered orally by gavage to mice on gene expression and secretion of murine IL-8 homologs (Cxcl1 and Cxcl2). MSM groups received (1) 25 mg/mL (corresponding to 5 mg of SM) or (2) 50 mg/mL (corresponding to 10 mg of SM) of MSM. MPL groups received (1) 96.2 mg/mL of MPL (corresponding to 5 mg of SM) or (2) 192.3 mg/mL of MPL (corresponding to 10 mg of SM). mRNA expression of (A) *Cxcl1* (KC) and (B) *Cxcl2* (MIP-2) in the ileum. The (C) portal plasma secretion and (D) systemic plasma secretion of CXCL1 (KC) and (E) portal plasma secretion (F) and systemic plasma secretion of CXCL2 (MIP-2) were performed by Luminex assay. Values represented the mean±S.E.M. Data were analyzed by two-way ANOVA (values bearing different superscript letters were significantly different) and the mean of each condition were compared with NaCl group (*P<.05 using post-hoc *vs* NaCl). Dotted lines represent the mean value of a NaCl group. Number of mice in each group is indicated within the bars. CXCL1, keratinocyte chemoattactant (KC); CXCL2, macrophage inflammatory protein-2α (Mip-2); MPL, milk polar lipids; MSM, milk-sphingomyelin.

in the intestinal lumen now deserves to be studied in animal models and humans. Importantly, circulating IL-8 has otherwise been proposed as a potential biomarker for the diagnosis of colorectal cancer because IL-8 is upregulated in such cancer and promotes tumor growth, invasion and metastasis [68]. Therefore, potential negative effects of exacerbated basolateral IL-8 secretion have to be considered. In this respect, previous studies in rat model of chemically-induced colon cancer reported beneficial effects of both pure SM [47,69] and total MPL [9], which inhibit colon cancer when incorporated in rat diet. However, deleterious impacts of pure MSM on a healthy intestine or on an inflamed intestine cannot be ruled out from our results. In this respect, the neutral impact of total MPL can be considered beneficial, as well as the lack of basolateral secretion of IL-8 with MPL.

Because SM is not directly absorbed as such by the cells, it would also be interesting to identify which of its metabolites could impact the gut barrier. The question is relevant as a study reported that the addition of exogenous sphingomyelinase, inducing hydrolysis of SM to ceramides, increases intestinal epithelial cell permeability [70]. Moreover, we previously observed that Caco-2/TC7 cells express both alkaline sphingomyelinase (ENPP7) and phospholipase A2 (PLA2G6), indicating ability to hydrolyze to some extent the incubated SM and PL. In the present study, C16-Cer did not impact tight junction and IL-8 expression but other concentrations of Cer-C16 or other types of ceramides should now be tested. Indeed in a different cell type namely macrophages, ceramides (C16 and C24) and MSM were reported to present similar blunting effects on LPS stimulation of macrophages in vitro [42]. Moreover in these macrophages, sphingosine/sphingoid base but not FA composition was found to be an important trigger of their effects [42]. Our results in Caco-2/TC7 cells

also support a limited impact of FA composition as ESM and MSM had similar effects on tight junction and IL-8 gene expressions. Because d18:1-sphingosine is the main sphingoid base in ESM and the most frequent sphingoid base in MSM [50], d18:1-sphingosine may be a common metabolite released after digestion that can be involved in both ESM and MSM impact on intestinal cells, which will deserve further investigation. A mouse gavage study with ESM (4 mg) resulted in increased expression of another tight junction protein, namely claudin-4 [71]. Therefore, the specific impacts of milk *vs* egg SM and the general impacts of dietary SM should be further explored *in vivo* with different amounts and feeding duration.

Among study limitations, MPL treatments were not isolipidic with MSM treatments as we favored to have iso-SM conditions either pure or contained in the total MPL. The impact of lower amounts of MPL and of each other MPL molecular species on gut barrier thus remain to be studied. Furthermore, the impact of increased concentrations of recombinant IL-8 on tight junction expression in Caco-2 cells should be further studied as well as using different types of control conditions for cells. The gene expression of tight junction proteins was impacted by SM and rh IL-8 but their effect on the amount of tight junction proteins after different doses and treatment durations remains to be quantified both in vitro and *in vivo*. Moreover, the impact of SM and its metabolites on the response of intestinal cells to LPS remains to be elucidated. In this respect, here the permeability of Caco-2/TC7 cells to macromolecules was not affected but this should now be tested in other models such as those of altered gut barrier and/or intestinal inflammation. This could be performed *e.g.* using permeability markers of different molecular weight and including labeled LPS markers for LPS translocation assays. Notably, in a mouse model of colitis, 4 mg/day of ESM had detrimental effects on colitis phenotype, opening questions regarding translation to Crohn's disease [72]. Therefore, MSM *vs* MPL impact in such disease models should now be clarified.

In conclusion, results of the present study indicate that Caco-2/TC7 cell incubation with mixed lipid micelles enriched with dietary SM (from milk but also egg) increases the expression of tight junction and induces a substantial increase of IL-8 secretion. Therefore, SM did not disrupt the intestinal barrier and it also induced early signs of barrier stimulation. Dietary SM could thus present an interesting effector to regulate intestinal permeability and impact on tight junction can be explained by a specific action of IL-8. Longer-term consequences of SM and IL-8 on tight junctions and barrier function will now have to be established. Moreover, healthcare professionals can need clinical nutrition products including bioactive ingredients for primary prevention or for therapeutic purposes. A recent large epidemiological study reports that milk consumption may be associated with a decreased risk of developing Crohn's disease, an intestinal disease characterized by impaired gut barrier or so-called leaky gut [73]. Therefore, it will be important in the future to evaluate and understand the possible properties of MSM as a compound involved in a decrease of permeability which could present interesting protective effects in obesity [11,74] or in leaky gut diseases.

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Statement of authors' contributions to manuscript

The authors' responsibilities were as follows – M.M., A.P., F.L. and M.-C.M. designed the research; M.M. and M.-C.M. wrote the manuscript; M.M., A.P., A.D., C.B., E.M., E.L., K.B., F.J., D.C., L.G., S.V., F.L., and M.-C.M. conducted the research; M.M. performed the statistical analysis; A.D. provided support for in vitro experiments and C.B. provided support for *in vivo* experiments; A.D. and C.B. provided technical support for analysis; E.L. and E.M. made qPCR analyses. K.B. and F.J. provided essential material for research and collaborate for lipids analyses. D.C. provided SM molecular species profile/FA distribution; L.G. and S.V. performed plasma biochemical analysis of inflammatory markers using Luminex. A.P., C.B., E.M., S.V., F.L. reviewed the manuscript. All authors: read and approved the final manuscript. M.-C.M has final responsibility for manuscript.

Conflict of interest statement

This study was funded by CNIEL (French Dairy Interbranch Organization) (SMILE project). MCM received other research funding on other topics from Sodiaal-Candia R&D, CNIEL and Nutricia Research. MCM has consultancy activities for food & dairy companies. These activities had no link with the present study. MCM coordinated a research project funded by the National Research Agency on the valuing and health properties of milk polar lipids (VALOBAB ANR-2011-0007, 2012–2016). FJ and KB are employees of ITERG (Fats, oils and crops Interbranch Technical Institute). FL and MM participated in a research project on another topic funded by Sodiaal-Candia R&D.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnutbio.2018.12.007.

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