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1 **Running Title: *Spingomyelin impacts intestinal tight junction expression***

2 **Acute effects of milk polar lipids on intestinal tight junction expression:**

3 **towards **an impact** of sphingomyelin through the regulation of IL-8 secretion?**

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25 **Abstract**

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

44

45 **Key words for publication:** Caco-2; Phospholipid; Milk Fat Globule Membrane; Dairy; Gut
46 barrier; Cytokine.

47 **1. Introduction**

48 Milk and dairy products are important nutritious food worldwide. The International Dairy
49 Federation estimated the average consumption of dairy products in the world to 111 kg in
50 2016 per year and per person. Bovine milk contains 3.5 to 5% of lipids and fat is present in
51 milk as milk fat globules, comprising a core of triglycerides (TG, ~98%) surrounded by their
52 milk fat globule membrane (MFGM). MFGM is a tri-layered biological membrane rich in
53 bioactive phospholipids/polar lipids (PL) (~0.5 to 1%) [1]. Dairy products are thus a natural
54 source of PL, which have interesting natural functional properties for the food industry as
55 emulsifiers [2] and may exert beneficial effects on health [3-5]. Interest has also recently
56 grown on using milk polar lipids (MPL) as potential nutraceutical, notably via rich sources
57 such as buttermilk [3]. Indeed, several rodent studies have demonstrated that MPL are able to
58 decrease hepatic lipid accumulation [6] and intestinal cholesterol absorption [7]. MPL have
59 also beneficial properties to modulate lipid absorption and postprandial lipemia [8] and to
60 inhibit the growth of cancer cells [9]. MPL have a specific PL profile with a large proportion
61 of SM (~24%), in comparison with other sources of PL products. Indeed, soybean PL are
62 devoid of SM and egg PL contains ~1.5% of SM [10]. A recent study in mice reported that
63 milk-SM (MSM) had a beneficial impact on hepatic lipid accumulation, decreased muscle
64 inflammation and lowered pro-inflammatory endotoxemia [11], which is one reported
65 consequence of altered gut barrier [12, 13]. Moreover in comparison with soybean PL (other
66 source of PL), MPL supplementation can induce (i) lower expression of markers of
67 inflammation and of macrophage infiltration in epididymal adipose tissue and (ii) more goblet
68 cells in colon [14]. Furthermore, in rat pups MPL [15] and MSM [16] are implicated in
69 intestinal development. For example, villi, crypts and Paneth cells are similar after MPL-
70 based milk formula and after mother milk, while they are altered after vegetable fat-based
71 milk formula [15]. Altogether, these results suggest various effects of MPL on the intestine, a

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

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

97 inflammatory protein-2 α (Mip-2) (CXCL2) are suggested to be functional homologs of IL-8
98 [32, 33].

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

103

104 2. Methods

105 2.1. MPL preparation

106 MSM (bovine) and egg-sphingomyelin (ESM, chicken) were purchased at >99% purity from
107 Avanti Polar Lipids, Inc (Alabaster, AL, USA). C16-Ceramide (C16-Cer, d18:1/16:0) was
108 purchased at 98% purity from Sigma (Saint-Quentin-Fallavier, France). The MPL-rich
109 ingredient was a phospholipid concentrate from butterserum (most MPL-rich type of
110 buttermilk obtained from anhydrous milkfat production), kindly provided by Corman
111 (Limbourg, Belgium). It was purified using Folch extraction followed by two subsequent
112 acetone extractions. The extract of MPL-rich ingredient contained >78% of PL, the balance
113 consisting of TG. A detailed PL profile is available in Table 1 and is consistent with the
114 literature [34]. The SM profile of MSM, ESM and MPL-rich ingredient (Fig. S1) was
115 analyzed by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) as previously
116 described [35]. As expected, the FA species of ESM had less diversity than in MSM and
117 mostly composed of C16; in turn the FA profile of commercial pure MSM and of SM within
118 MPL was similar.

119 2.2. *In vitro*

120 2.2.1 *Culture preparation*

121 Caco-2/TC7 intestinal cells, provided by Dr. Rousset (INSERM U505, Paris, France), derived
122 from a human adenocarcinoma is able to undergo differentiation into polarized epithelial cells
123 that show a brush border phenotype and form well-developed and functional tight junction
124 complexes [36]. This clone, compared to the parental cell line, increased expression of
125 markers associated with differentiation of enterocytes [37]. They were routinely cultured in
126 high-glucose (4.5 g/L) medium with glutamine (DMEM GlutaMAX™; Invitrogen, Thermo
127 Fisher Scientific, Waltham, MA, USA) supplemented with 20% fetal calf serum (v/v), 1% of
128 nonessential amino acids (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) and 1%
129 antibiotics (penicillin/streptomycin; Invitrogen, Thermo Fisher Scientific, Waltham, MA,
130 USA), and maintained under a 10% CO₂ atmosphere at 37°C. Cells were seeded at a density
131 of 2.5 x 10⁵ cells per Transwell filter inserts (12 mm polycarbonate, 0.4 µm pore size Costar,
132 Cambridge, MA, USA) in 6-well plates. The experiments were performed after 21 days of
133 culture on semi-permeable filters. Prior to lipid treatment, cells were incubated with serum-
134 free complete medium for 24 h. The incubation media containing so-called “mixed micelles”,
135 mimicking postprandial lipids in the gut during digestion [38], were prepared as follows: oleic
136 acid (0.5 mM), 2-monooleylglycerol (0.2 mM), cholesterol (0.05 mM), taurocholic acid (2
137 mM), L- α -lysophosphatidylcholine (0.2 mM), phosphatidylcholine (0.4 mM). For the
138 preparation of lipid micelles, stock solutions were prepared in chloroform-methanol.
139 Appropriate volumes were added in glass tube, dried under a stream of nitrogen. Lipid
140 mixtures were frozen at -20°C until the experiment. The day of the experiment, lipids were
141 vortexed in serum-free complete medium and further dispersed in an ultrasonic bath for 30
142 min at 37°C. Cells were incubated during 24h with micelle-containing medium into the apical

143 media and the basolateral compartment received serum-free complete medium complemented
144 with 1% nonessential amino acids, 1% pyruvate and 1% antibiotics.

145 Different experiments were conducted in which: (i) MPL-rich ingredient or MSM were added
146 at the corresponding concentration of 0.2 or 0.4 mM of SM into the micelles in culture
147 medium (i.e. considering average molecular weight of pure MSM: 0.16 or 0.31 mg/mL). PC
148 was added to equilibrate micelles at 0.4 mM. Analyses were performed on three independent
149 experiments; (ii) MSM was added into the micelles at the corresponding concentration of 0.2,
150 0.4 and 0.6 mM in culture medium. mRNA levels were performed on three independent
151 experiments and IL-8 secretion were performed on five independent experiments. For 0.6 mM
152 condition, only two independent experiments were performed. All experiments were realized
153 in triplicate. Of note, our rationale for the tested SM proportions within lipids incubated onto
154 Caco-2 cells was elaborated in a nutraceutical approach. Our previous analyses showed that
155 SM content in MPL-rich products, buttermilk and butterserum, was 5-13% of total lipids [35].
156 Moreover, it has been reported that 20% of MSM would remain intact during human
157 digestion [39] while only <1% of FA from other lipids (TAG, PL) remain unabsorbed [40].
158 Therefore, we calculated the expected molar ratio of intact SM to free FA to be in the order of
159 1:3 from buttermilk to 1:1 from butterserum. The amount of SM that we incorporated in
160 model micelles corresponds to this range of SM:FA ratios.

161 To evaluate if a digestion byproduct of MSM can induce similar effects like intact MSM, we
162 performed similar incubations with micelles enriched with 0.2 or 0.4 mM of C16-Cer, the
163 major form of ceramide derived from MSM found in human ileostomy [39]. Cells were
164 incubated during 24h and mRNA levels were performed on one independent experiment in
165 triplicate. To evaluate if the impact of MSM is due to the specific FA and sphingoid base
166 composition of milk, we evaluated the impact of ESM on tight junction. Mixed micelles were
167 supplemented with 0.2 or 0.4 mM of MSM or ESM in culture medium (i.e. considering

168 average molecular weight of pure ESM: 0.14 or 0.29 mg/mL). Cells were incubated during
169 24h and mRNA levels were performed on three independent experiments.

170 We verified that the size of incubated lipid structures was not dramatically altered by
171 differences in lipid composition using Dynamic Light Scattering (Zetasizer NanoS, Malvern,
172 UK). “Mixed lipid micelles” media contained both structures of micellar size (in the range 8-
173 40 nm) and of lipid vesicle size (in the range 150-300 nm). Importantly, this is altogether
174 consistent with the size of lipid structures reported in duodenal lipid digestion content in
175 humans [38, 41] and was similar regardless of micelle composition. The size of micelles
176 *stricto sensu* was similar regardless of inclusion of milk or egg SM.

177 Another experiment was conducted; recombinant human IL-8 was incubated during 24h at
178 100 pg/mL on apical media and/or 80 pg/mL on basolateral media. These concentrations were
179 used to mimic the previously observed concentrations in apical and basolateral media after
180 MSM incubation. mRNA levels were performed on one experiment performed in triplicate.

181 Recombinant human Interleukin-8 human (rh IL-8) was obtained at >98% purity from Sigma
182 (Saint-Quentin-Fallavier, France).

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

186

187 2.2.2 Measurement of paracellular permeability

188 Cellular permeability was evaluated for cells incubated with total MPL or pure MSM by
189 measuring the emitted fluorescence at 530 nm for FITC-Dextran-4 (excitation at 485 nm) and
190 at 520 nm for Lucifer Yellow (excitation at 490 nm). In brief, 1 mg/mL of FITC/dextran
191 (Sigma, Saint-Quentin-Fallavier, France) or 45.7 µg/mL of Lucifer Yellow (Sigma, Saint-
192 Quentin-Fallavier, France) were added in the apical side of the transwell chamber. Medium

193 from the basolateral side was collected, and fluorescence intensity was measured using a
194 fluorescence spectrophotometer (SAFAS Xenius XL, SAFAS Monaco, France) after 120
195 minutes.

196

197 *2.2.3 Immunofluorescence*

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

206

207 *2.2.4 Quantification of cytokine secretion*

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

213

214 *2.3. In vivo*

215 *2.3.1. Animals and experimental protocol*

216 C57BL/6 mice (male, 19.4±0.2g, 8-weeks-old; Janvier SA, Saint Berthevin, France) were
217 housed 5 per cage in a temperature-controlled room (24 ± 1°C, 12 hour daylight cycle, free

218 access to food and water). After two weeks of acclimatization with a chow diet, they were
219 randomly divided into groups of 10 mice (5 mice/cage). On the experimental day, after
220 overnight fasting, mice received by oral gavage, diluted in NaCl:

- 221 - **MSM 1:** 200 μ L of 25 mg/mL of MSM (corresponding to 5 mg)
- 222 - **MSM 2:** 200 μ L of 50 mg/mL of MSM (corresponding to 10 mg)
- 223 - **MPL 1:** 200 μ L of 96.2 mg/mL of MPL (corresponding to 5 mg of SM)
- 224 - **MPL 2:** 200 μ L of 192.3 mg/mL of MPL (corresponding to 10 mg of SM).

225 **Of note, using the Human Equivalent Dose calculation of the FDA [42], 5 mg and 10 mg of**
226 **SM in mice would correspond to a bolus of 0.8 g and 1.6 g for a human of 60 kg body**
227 **weight, respectively, i.e., the amount within an intake of 0.3 to 0.6 L of butterserum.**

228 A group received saline only and was used as control. Mice were euthanized 4h after gavage.
229 For euthanizing, mice were anaesthetized using isoflurane (4% isoflurane in 100% O₂ until
230 unconscious and anaesthesia was maintained with 2% isoflurane in 100% O₂). After
231 laparotomy, blood was collected by portal vein with heparinized syringes and mice were
232 killed after a terminal blood collection by cardiac puncture. Intestines were collected after a
233 wash of the lumen with PBS. Whole sections of intestine were immediately frozen in liquid
234 nitrogen and stored at -80°C before analysed by RT-qPCR. All procedures were approved by
235 the Animal Ethic Committee of University Lyon 1.

236

237 2.3.2. Plasma biochemical analysis

238 Plasma samples from cardiac puncture and portal vein were obtained after centrifugation
239 (3600 g, 15 min, 4°C). Plasma concentrations of IL6, TNF- α , IL-1 β , IL-10, MCP-1, CXCL1
240 (limit of detection: 6pg/mL) and CXCL2 (limit of detection: 160 pg/mL) were determined
241 using a multiplex immunoassay kit (Milliplex® MAP Mouse Cytokine, Mcytomag-70K,
242 Merck Millipore) and measured using a Bioplex 200 system (Biorad, CA, USA).

243 2.4. RNA extraction and quantitative real-time PCR

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

255

256 2.5. Statistical analysis

257 All data, presented as means \pm standard error of the mean (SEM), were analysed with Graph
258 Pad Prism Software (version 6.01, San Diego, CA, USA). Normality of data was examined
259 using Shapiro-Wilk test. One-way analyses of variance (ANOVA) were performed on normal
260 data. For non-normal data or small number of observations, a Kruskal-Wallis test was
261 performed followed by a Dunn post hoc test. Two-way ANOVA were used to assess whether
262 there were significant differences between source of SM (pure or in total MPL) within dose
263 (values bearing different superscript letters were significantly different). When the interaction
264 term was not significant this analysis was followed by Bonferroni post hoc multiple
265 comparisons test. If the interaction term was significant an unpaired test was performed to
266 determine differences between MSM and MPL groups. One-way ANOVA and a *post hoc*
267 Dunnett test were performed to assess statistical differences from control (mixed micelles

268 without SM or NaCl group in the *in vivo* study) (an asterisk represents a significant difference
269 with control).

270

271 3. Results

272 3.1. Pure sphingomyelin in model postprandial lipid micelles increases the gene
273 expression of tight junction proteins and induces IL-8 secretion in Caco-2/TC7 cells

274 Because MPL contain ~24% of MSM, we investigated impact of pure MSM vs total MPL on
275 Caco-2/TC7. Increasing concentration of MSM or MPL did not affect Caco-2 TEER after 24h
276 incubation (data not shown). As shown in Figure 1, the SM source (pure MSM or MPL
277 including MSM at the same dose) had a significant impact on the gene expression of a
278 transmembrane protein of tight junctions, the JAM-1 (Fig. 1B, $P_{\text{source}} < 0.05$). The gene
279 expression of Occludin, another transmembrane protein, and ZO-1, an intracellular protein,
280 were significantly higher for 0.4 mM of MSM compared to other groups, these effects being
281 specific of pure MSM (Fig. 1A, C). In comparison with control mixed micelles devoid of
282 MSM, we observed a significant increase of mRNA level of Occludin ($P < 0.05$ for 0.2 mM of
283 MSM and $P < 0.0001$ for 0.4 mM of MSM) and ZO-1 ($P < 0.05$ for 0.2 mM of MSM and
284 $P < 0.0001$ for 0.4 mM of MSM) (Fig. 1A, C). No difference was observed when cells were
285 incubated with mixed micelles supplemented with total MPL. Compared with control mixed
286 micelles devoid of SM, pure MSM increased significantly the gene expression and secretion
287 of IL-8 ($P < 0.05$ for 0.2 mM of MSM and $P < 0.01$ for 0.4 mM of MSM) while total MPL did
288 not (Fig. 1D, E), despite containing the same SM amount. IL-8 secretion increased in the
289 apical compartment with pure MSM ($P < 0.05$ for 0.4 mM of MSM) and also in the basolateral
290 compartment ($P < 0.05$ with 0.2 mM and $P < 0.05$ with 0.4 mM of pure MSM in comparison
291 with mixed micelles devoid of SM). Total MPL incorporated in mixed micelles did not induce
292 a significant release of IL-8 (Fig. 1D, E), confirmed by a source dependent effect for IL-8

293 secretion (Fig. 1D, $P_{\text{source}} < 0.01$) and for the mRNA expression (Fig. 1E, $P_{\text{source}} < 0.01$). Of
294 note, gene expression of another inflammatory marker, MCP-1, was not impacted by MSM
295 (results not shown). Moreover, isolipidic lipid micelles containing increasing proportions of
296 MSM confirmed that the impact of MSM on tight junction proteins and IL-8 observed above
297 was due to increased amount of MSM and not to the higher total lipid content of the micelles
298 following addition of MSM (data not shown). A major digestion product of MSM, namely
299 C16-Cer, did not induce all the above-mentioned impacts on Caco-2/TC7 cells (Fig. S2). Of
300 note, effects of SM were not specific of milk source. Indeed, we also observed highest gene
301 expression of tight junction protein and an increase on IL-8 secretion and expression with
302 ESM (Fig. S3) which differs from MSM by the FA (Fig. S1) and sphingoid base composition
303 [43].

304

305 *3.2. Sphingomyelin induces a dose-dependent secretion of IL-8 that can be a possible effector*
306 *of the impact on the gene expression of tight junction proteins*

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

314 A complementary study was performed to test the dose-response impact of MSM on Caco-
315 2/TC7 cells from 0.2 to 0.6 mM. Of note, control micelles devoid of MSM did not have a
316 different impact on the expression of tight junction proteins than pure culture medium (dotted
317 line). There was a significant increase in gene expression of Occludin with 0.4 mM of MSM

318 (Fig. 2A, $P<0.05$). For JAM-1, incubation with micelles containing MSM led to an increase
319 of mRNA expression for MSM 0.4 mM (Fig. 2B, $P<0.05$). The gene expression of ZO-1 was
320 also significantly higher for MSM 0.4 mM ($P<0.01$) and MSM 0.6 mM ($P<0.05$) compared to
321 control (mixed micelles devoid MSM) (Fig. 2C). The mRNA level of Claudin-1 was no
322 different between treatments (Fig. 2D). Both in the apical and basolateral compartments,
323 dose-response secretions of IL-8 were observed after apical stimulation with the micelles
324 containing MSM (Fig. 2E) (Fig. 2E shows Spearman correlations between MSM concentration
325 and IL-8 secretion). IL-8 became significantly different from the control lipid micelles for the
326 0.6 mM treatment in both compartments ($P<0.05$). Gene expression of IL-8 increased
327 significantly for MSM 0.4 mM ($P<0.05$ vs control, Fig. 2F). As shown in Figure 2G, there
328 was a positive correlation between apical and basolateral IL-8 concentrations ($r=0.94$,
329 $P<0.0001$) suggesting a common mechanism. This SM-induced secretion was specific of IL-8
330 as we observed no impact on IL-6, IL-17, IL-22, TNF- α and MCP-1 secretions (data not
331 shown) and no differences on the gene expression of IL-1 β and MCP-1 (Fig. S4D, E).

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

339

340 3.3. Milk sphingomyelin and total milk phospholipids can both increase the gene expression
341 of IL-8 homologs in ileum *in vivo* in mice

342 We finally performed a study in mice orally force-fed with MPL or MSM to evaluate their
343 impacts *in vivo*. We specifically examined the expression of murine IL-8 homologs, *Cxcl1*
344 (KC) and *Cxcl2* (Mip-2), in jejunum and ileum of mice. The SM source (pure MSM or MPL
345 including MSM at the same dose) had a significant impact on the gene expression of *Cxcl1* in
346 the ileum (Fig. 4A, $P_{\text{source}} < 0.05$). *Cxcl1* and *Cxcl2* mRNA expressions were upregulated in the
347 MSM1 mouse ileum compared with mice forced-fed with NaCl (Fig. 4A, B, $P < 0.01$). We also
348 observed an overexpression of *Cxcl2* for MPL1 mice in comparison with NaCl mice (Fig. 4B,
349 $P < 0.05$). However, no significant impact was observed in jejunum for *Cxcl1* (KC) and *Cxcl2*
350 (Mip-2) (Fig. S5). The concentration of IL-8 homologs showed no differences among groups
351 in the portal vein (directly derived from the intestine) nor systemic circulation (Fig. 4C-F). Of
352 note, IL-1 β , TNF- α , IL-6, IL-10 and MCP-1 secretions were indistinguishable in portal vein
353 and systemic circulation (data not shown). Altogether, the outcome most impacted by MPL
354 and MSM was *Cxcl2* expression in the ileum. Moderate effects on tight junction were
355 observed (Fig. S6). Indeed, the SM source had a significant impact on the gene expression of
356 *Occludin* (Fig. S6A, B, $P_{\text{source}} < 0.05$ in the duodenum and jejunum) and *Zo-1* (Fig S6G, I,
357 $P_{\text{source}} < 0.05$ in the duodenum and ileum).

358

359 4. Discussion

360 The gastrointestinal tract is a complex interface between the external environment and
361 the immune system, establishing a dynamic barrier that enables the absorption of dietary
362 nutrients and the exclusion of potentially harmful compounds from the intestinal lumen. The
363 expression of tight junction proteins, implicated in intestinal permeability and barrier function
364 [44], can be regulated by dietary components [17]. Tight junctions are not static barriers but

365 highly dynamic structures that are constantly being remodeled due to interactions with
366 external stimuli, such as food residues and pathogenic and commensal bacteria. Up to date,
367 different studies examined the impact of the amount and FA profile of dietary fat on tight
368 junctions [18, 19]. Study showed that gangliosides, which are complex sphingolipids, can
369 protect tight junctions after an endotoxin challenge [45]. Short chain FA, especially butyrate
370 could impact tight junction permeability [46] and assembly of tight junction [47]. In this
371 context, the interest for such functional ingredient on a scientific point of view as well as for
372 the food industry purpose is growing. Dietary sphingolipids of milk origin, which are present
373 in the native complex MPL, could present benefits regarding the prevention of lipid
374 metabolism [6-8] and inflammation disorders [9, 14, 48]. Notably, this contributes to propose
375 MPL-rich sources such as buttermilk/butterserum as candidates for developing bioactive food
376 ingredients or nutraceuticals [3-5; 35]. However to date, little is known about the impact of
377 MPL and also of MSM on tight junctions. Norris et al., have observed that a high fat diet
378 enriched in MSM induced a decrease of endotoxemia in mice [11]. This protective effect
379 could be mediated by a mechanism of gut barrier enhancement. However, authors
380 demonstrated no impact of MSM on tight junction gene expression nor on paracellular
381 permeability in mice, while other reported increased colonic expression of *Occludin* and *ZO-1*
382 but no beneficial impact on gut permeability in a model of mice challenged with LPS [49].
383 *In vivo*, sphingolipids are considered to be highly bioactive compounds and their endogenous
384 metabolism can also be modified by constituents of the diet with consequences for cell
385 regulation and disease [50, 51]. Sphingolipid composition of biological membranes in the
386 body consists mainly of SM, as is also the case for MPL. Here we studied the links between
387 dietary SM (pure or within MPL) and the expression of tight junctions in acute *in vitro* and *in*
388 *vivo* studies. In Caco-2/TC7 cells, upon incubation with mixed lipid micelles containing pure
389 MSM or total MPL, an overexpression of tight junction proteins was observed with increased

390 concentrations of MSM but not with MPL (naturally containing equivalent amounts of
391 MSM). Of note, we verified that the global composition of SM species in pure MSM and in
392 MPL was similar. Therefore, it should now be elucidated whether this lack of impact of MPL
393 on tight junction expression is due to (i) specific effects of other MPL species, including
394 phosphatidyl-ethanolamine, -serine and -inositol (alone or synergistically, and possibly due to
395 their interfacial properties), (ii) a lower bioaccessibility of MSM for intestinal cells when
396 incorporated within MPL, or (iii) the higher total lipid concentration in MPL incubation
397 conditions vs MSM micelles. In MDCK cells, enhancement of tight junction barrier function
398 is correlated with higher expression of occludin [52]. Consistently, several studies have
399 shown a correlation between a decrease in occludin expression and an increase in epithelial
400 tight junction permeability, i.e. a decreased of function of gut barrier [53]. Therefore, the
401 increased expression of occludin with MSM in the present study can be considered as an early
402 signal of gut barrier enhancement, even if we did not observed any acute modification of
403 epithelial tight junction permeability in the present short-term conditions.

404 A possible molecular mechanism by which SM induced an increase in the expression of the
405 tight junction was then explored. Regulation of the assembly, disassembly, and maintenance
406 of tight junction structure is influenced by various physiological and pathological stimuli.
407 Cytokines, such as TNF- α , interferon gamma (IFN γ) [54], IL-4 and IL-13 [55] have been
408 reported to decrease barrier function [56]. Importantly, Motouri et al. showed that MSM plays
409 an important role in neonatal gut maturation during the suckling period [16]. Furthermore, IL-
410 8 present in human milk has been identified as a major actor responsible for the development
411 of the intestine. IL-8 has been shown to increase cell migration, proliferation and
412 differentiation when Caco-2 cells were treated with rh IL-8 *in vitro* [31]. We therefore emitted
413 a novel hypothesis regarding a link between dietary SM, IL-8 secretion and the intestinal
414 barrier, which had not yet been established in the literature. In this study, micelles containing

415 SM induced an overexpression of IL-8 and a dose-response vectorial secretion, which we
416 propose can be involved in the observed modulation of tight junction. It has been shown that
417 TNF- α induces IL-8 secretion and increases tight junction permeability [23]. However, in our
418 study, no impact of MSM on TNF- α nor MCP-1 and IL-1 β were observed. *In vivo*, IL-8 is
419 known for its involvement in the inflammatory process [29]. A basolateral secretion of IL-8
420 plays a role in the recruitment of circulating neutrophils from the bloodstream to the site of
421 tissue injury or infection. Moreover, it is speculated that apically secreted IL-8 may initiate or
422 augment the pathway responses in epithelial restitution before any potential loss of intestinal
423 barrier integrity because of toxin exposure [57-60]. Thus considering the involvement in the
424 development of the immature intestine of both IL-8 [31] and dietary SM [16], this could also
425 suggest a role of both SM and IL-8 in the morphology of the mature intestine.

426 We therefore aimed to explore whether IL-8 could be partly responsible for the modification
427 of tight junction expression by MSM (indirect impact of SM on tight junction via IL-8
428 secretion). We observed an overexpression of tight junction expression when cells were
429 incubated with recombinant human IL-8, suggesting that the impact of MSM can be partly
430 due to a specific action of IL-8. It is widely known that IL-8 is involved in inflammatory
431 bowel disease [61] and is a target for inflammatory disease treatments [62, 63]. However, in
432 this study, (i) no other tested inflammatory markers were impacted and (ii) we observed
433 higher gene expression of tight junction proteins, which are usually impaired in inflammatory
434 bowel diseases. Evaluating the intracellular processes involved in the modulation of intestinal
435 tight junctions and gut barrier by IL-8 will now be important to understand mechanisms
436 involved in intestinal permeability disorders and to assess the consistency of novel therapeutic
437 strategies for intestinal diseases. Notably, further studies should be performed by removing or
438 mutating IL-8 or its receptor in the Caco-2 cells (e.g. siRNA transfection assays) or develop
439 transgenic mice (e.g. CXCR1/2-KO) to verify if the effects of MSM would be maintained

440 (suggesting a direct effect on tight junctions) or blunted (suggesting an IL-8 mediated effect
441 on tight junctions only). Moreover, incubation of Caco-2/TC7 cells with recombinant human
442 IL-8 induced an increased gene expression of IL-8. Because ZO-1 has been shown to be
443 implicated in IL-8 signaling in breast cancer cells [64] and lung cells [65], such an indirect
444 regulation loop of IL-8 via ZO-1 in Caco-2/TC7 cells would now deserve to be studied.

445 Our *in vivo* study shows an overexpression of IL-8 homologs in the ileum when mice were
446 gavaged with MPL or MSM (at ~5 mg or 10 mg of SM) and moderate effects on tight
447 junction protein expressions in the gut. Intestinal secretion of interleukins had been
448 demonstrated in several studies. Indeed, intestinal epithelial cells could secrete IL-8 in healthy
449 human [59] and in Caco-2 cells [57]. Other epithelial cells could have a polarized secretion of
450 interleukins, such as IL-1, IL-6 and IL-8 [66-68]. The relative functional importance of apical
451 vs basolateral secretion requires further elucidation. Moreover, the impact of MSM on the
452 secretion of IL-8 in the intestinal lumen now deserves to be studied in animal models and
453 humans. Importantly, circulating IL-8 has otherwise been proposed as a potential biomarker
454 for the diagnosis of colorectal cancer because IL-8 is upregulated in such cancer and promotes
455 tumor growth, invasion and metastasis [69]. Therefore, potential negative effects of
456 exacerbated basolateral IL-8 secretion have to be considered. In this respect, previous studies
457 in rat model of chemically-induced colon cancer reported beneficial effects of both pure SM
458 [48, 70] and total MPL [9], which inhibit colon cancer when incorporated in rat diet.
459 However, deleterious impacts of pure MSM on a healthy intestine or on an inflamed intestine
460 cannot be ruled out from our results. In this respect, the neutral impact of total MPL can be
461 considered beneficial, as well as the lack of basolateral secretion of IL-8 with MPL.

462 Because SM is not directly absorbed as such by the cells, it would also be interesting to
463 identify which of its metabolites could impact the gut barrier. The question is relevant as a
464 study reported that the addition of exogenous sphingomyelinase, inducing hydrolysis of SM

465 to ceramides, increases intestinal epithelial cell permeability [71]. Moreover, we observed that
466 Caco-2/TC7 cells express both alkaline sphingomyelinase (ENPP7) and phospholipase A2
467 (PLA2G6), indicating ability to hydrolyze to some extent the incubated SM and PL. In the
468 present study, C16-Cer did not impact tight junction and IL-8 expression but other
469 concentrations of Cer-C16 or other types of ceramides should now be tested. Indeed in a
470 different cell type namely macrophages, ceramides (C16 and C24) and MSM were reported to
471 present similar blunting effects on LPS stimulation of macrophages *in vitro* [43]. Moreover in
472 these macrophages, sphingosine/sphingoid base but not FA composition was found to be an
473 important trigger of their effects [43]. Our results in Caco-2/TC7 cells also support a limited
474 impact of FA composition as ESM and MSM had similar effects on tight junction and IL-8
475 gene expressions. Because d18:1-sphingosine is the main sphingoid base in ESM and the
476 most frequent sphingoid base in MSM [51], d18:1-sphingosine may be a common metabolite
477 released after digestion that can be involved in both ESM and MSM impact on intestinal cells,
478 which will deserve further investigation. A mouse gavage study with ESM (4 mg) resulted in
479 increased expression of another tight junction protein, namely claudin-4 [72]. Therefore, the
480 specific impacts of milk vs egg SM and the general impacts of dietary SM should be further
481 explored *in vivo* with different amounts and feeding duration.

482 Among study limitations, MPL treatments were not isolipidic with MSM treatments as we
483 favored to have iso-SM conditions either pure or contained in the total MPL. The impact of
484 lower amounts of MPL and of each other MPL molecular species on gut barrier thus remain
485 to be studied. Furthermore, the impact of increased concentrations of recombinant IL-8 on
486 tight junction expression in Caco-2 cells should be further studied as well as using different
487 types of control conditions for cells. The gene expression of tight junction proteins was
488 impacted by SM and rh IL-8 but their effect on the amount of tight junction proteins after
489 different doses and treatment durations remains to be quantified both *in vitro* and *in vivo*.

490 Moreover, the impact of SM and its metabolites on the response of intestinal cells to LPS
491 remains to be elucidated. In this respect, here the permeability of Caco-2/TC7 cells to
492 macromolecules was not affected but this should now be tested in other models such as those
493 of altered gut barrier and/or intestinal inflammation. This could be performed e.g. using
494 permeability markers of different molecular weight and including labeled LPS markers for
495 LPS translocation assays. Notably, in a mouse model of colitis, 4 mg/day of ESM had
496 detrimental effects on colitis phenotype, opening questions regarding translation to Crohn's
497 disease [73]. Therefore, MSM vs MPL impact in such disease models should now be
498 clarified.

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

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529 **Statement of authors’ contributions to manuscript**

530 The authors’ responsibilities were as follows – M.M., A.P., F.L. and M.-C.M. designed the
531 research; M.M. and M.-C.M. wrote the manuscript; M.M., A.P., A.D., C.B., E.M., E.L., K.B.,
532 F.J., D.C., L.G., S.V., F.L., and M.-C.M. conducted the research; M.M. performed the
533 statistical analysis; A.D. provided support for *in vitro* experiments and C.B. provided support
534 for *in vivo* experiments; A.D. and C.B. provided technical support for analysis; E.L. and E.M.
535 made qPCR analyses. K.B. and F.J. provided essential material for research and collaborate
536 for lipids analyses. **D.C. provided FA distribution of SM**; L.G. and S.V. performed plasma
537 biochemical analysis of inflammatory markers using Luminex. A.P., C.B., E.M., S.V., F.L.

538 reviewed the manuscript. All authors: read and approved the final manuscript. M.-C.M has
539 final responsibility for manuscript.

540 **Conflict of interest statement**

541 This study was funded by CNIEL (French Dairy Interbranch Organization). MCM received
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Figure Caption

Figure 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 \pm SEM (Three independent experiments in triplicate). Data were analysed 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 <0.05 using *post-hoc* vs 0mM). 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.

Figure 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 analysed 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 0mM MSM \pm SEM. **Data were analysed by one-way ANOVA** (* P <0.05, ** P <0.01, *** P <0.001 and **** P <0.0001). 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.

Figure 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 24h. Gene expression of (A) occludin, (B) JAM-1, (C) ZO-1 (D) and IL-8 were performed. Values represented the mean \pm SEM within one experiment (One experiment in triplicate). An asterisk represents a significant difference with DMEM condition at $P < 0.05$. IL-8, Interleukin-8; JAM-1, junction adhesion molecule 1; TBP, TATA-binding protein; Zo-1, Zonula occludens-1.

Figure 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 \pm SEM. Data were analysed by two-way ANOVA (values bearing different superscript letters were significantly different) and the mean of each condition were compared with NaCl group ($*P < 0.05$ 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 chemoattractant (KC); CXCL2, macrophage inflammatory protein-2 α (Mip-2); MPL, milk polar lipids; MSM, milk-sphingomyelin.

Figure 1.

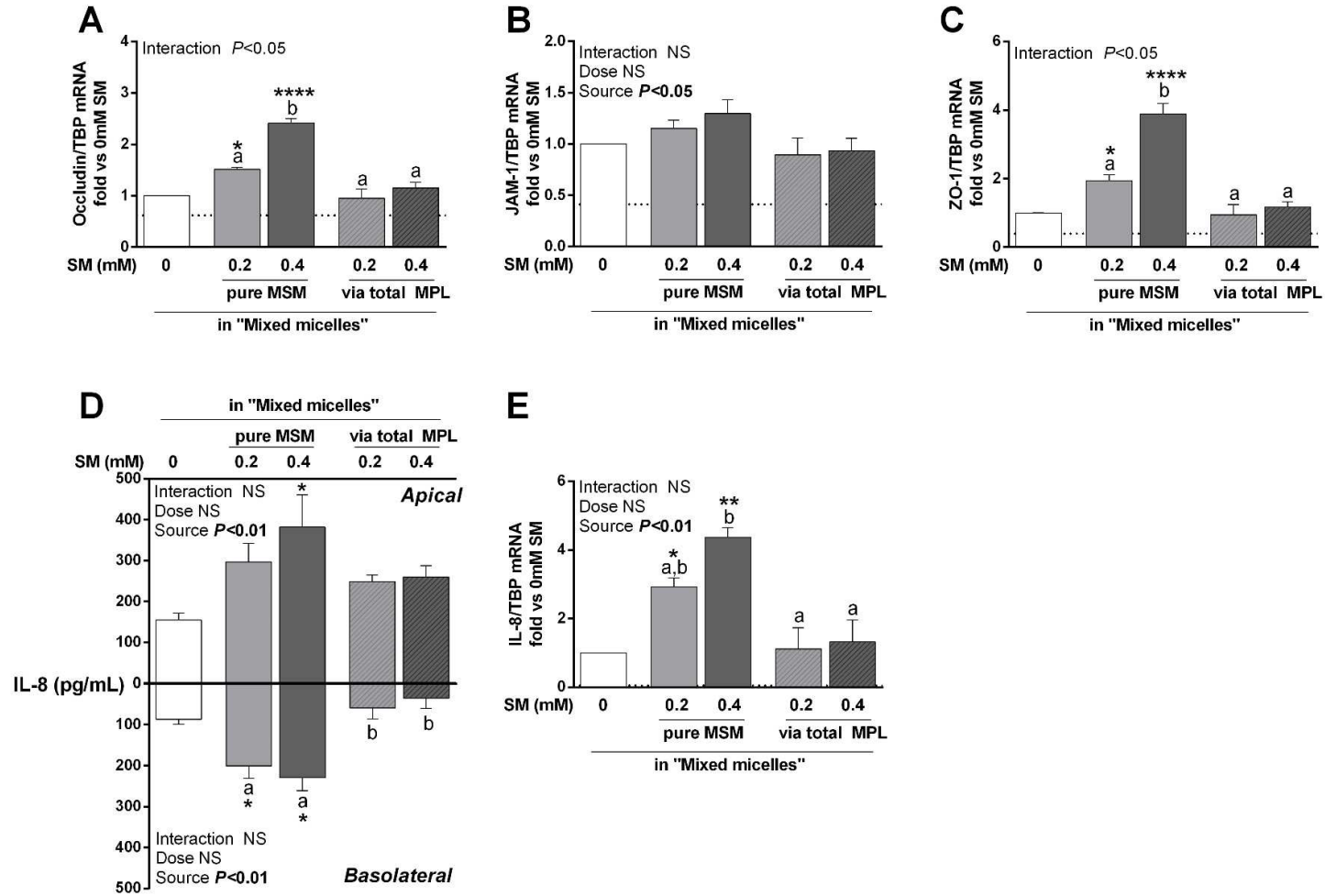


Figure 2.

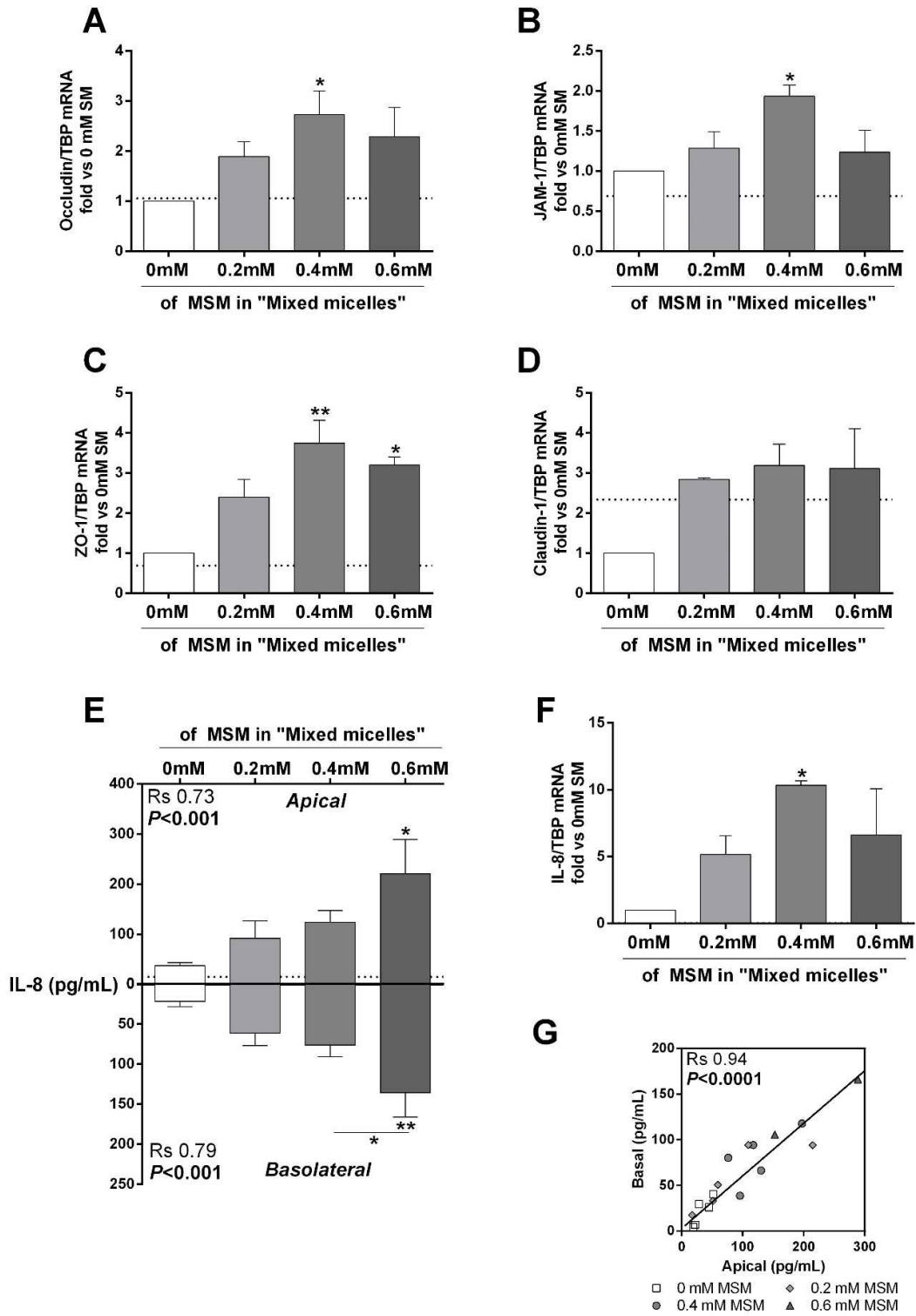


Figure 3.

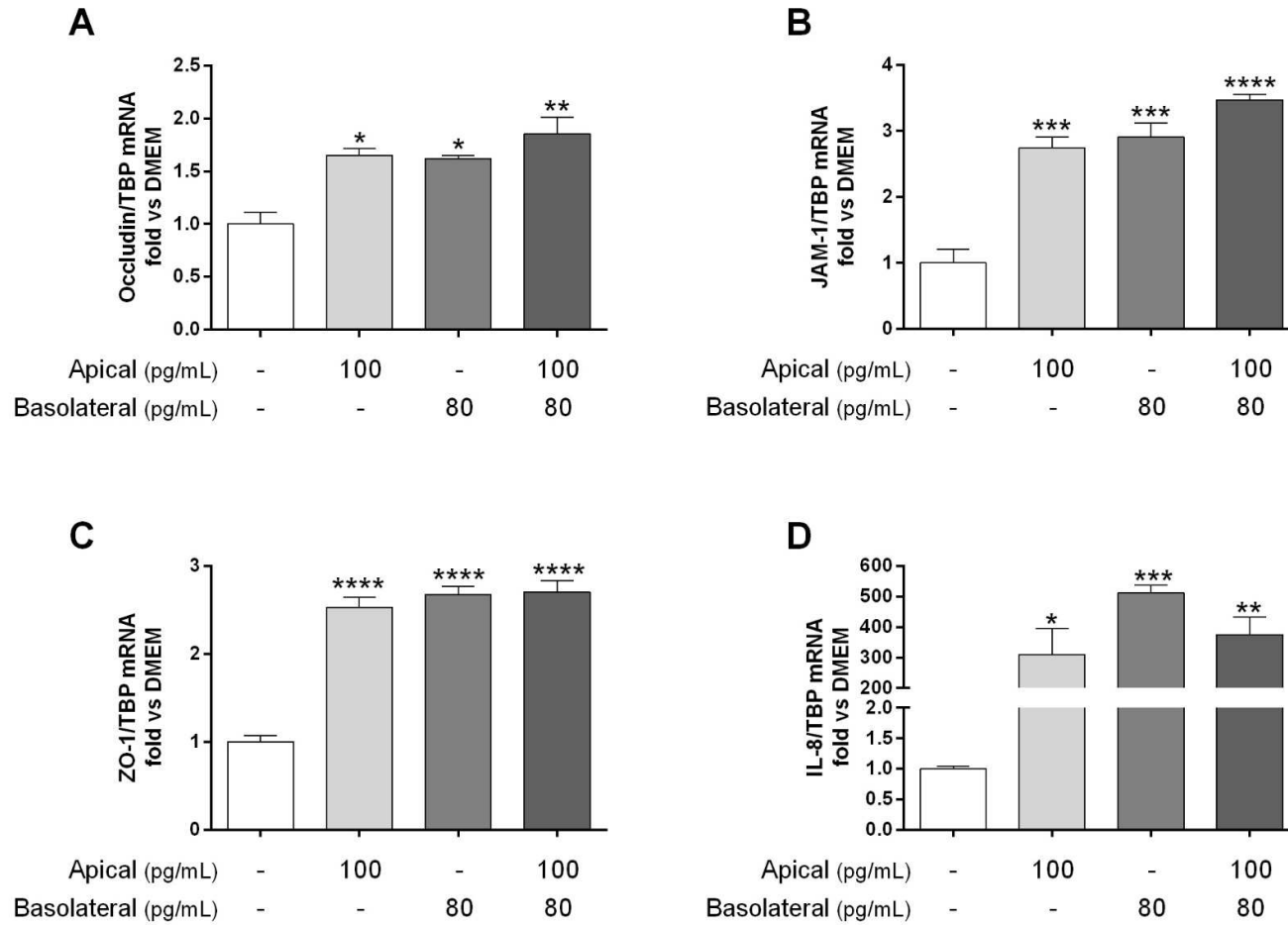


Figure 4.

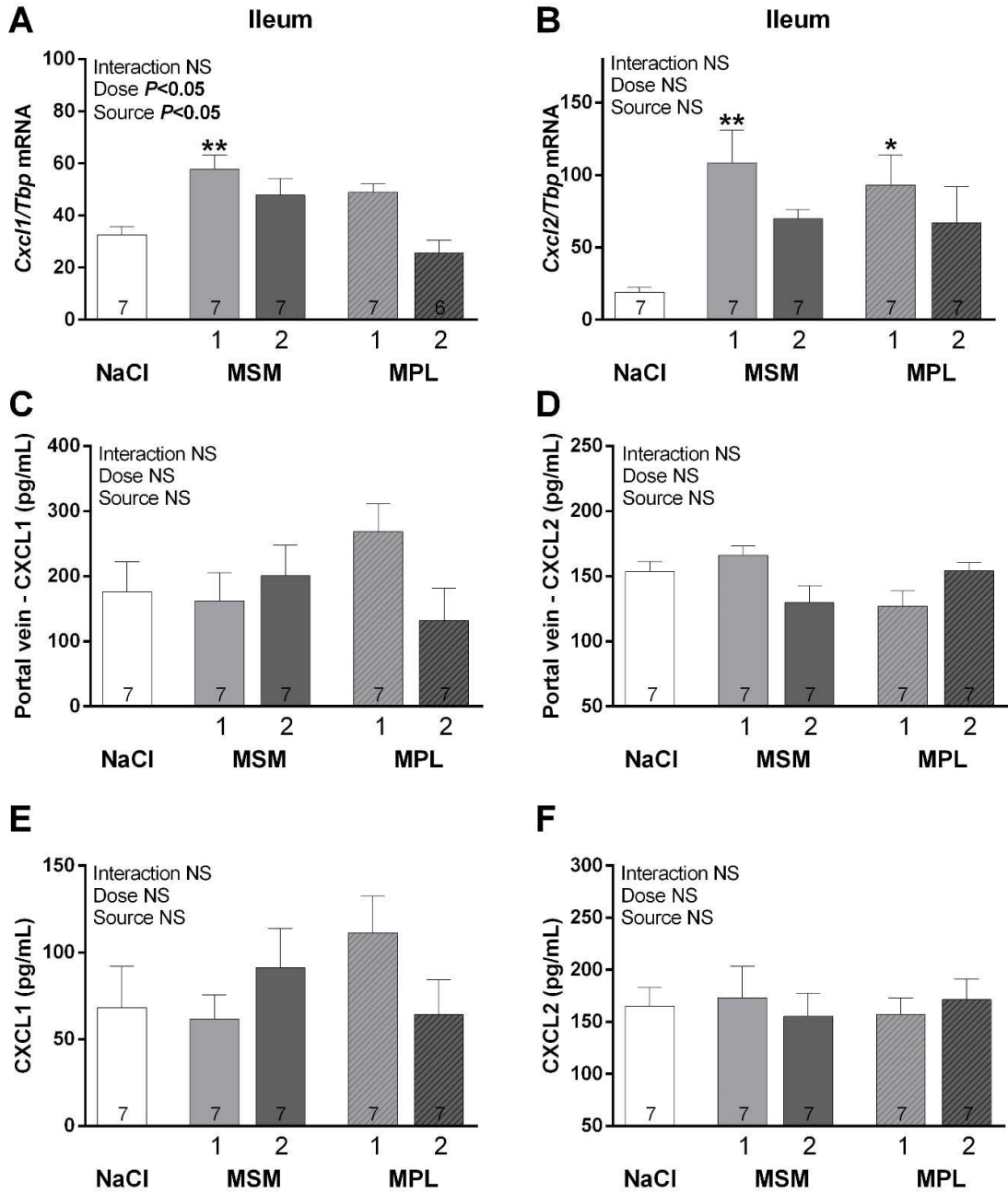


Table 1. Polar lipid profile of the MPL-rich ingredient used to *in vitro* and *in vivo* studies.¹

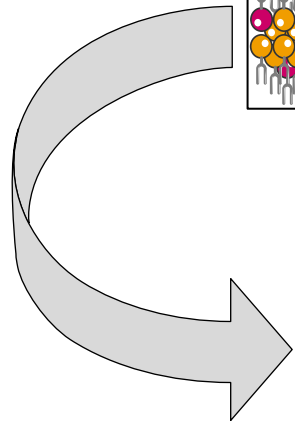
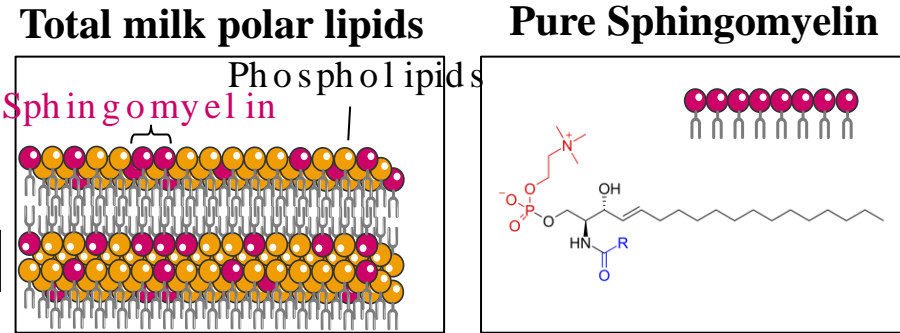
PL classes, % total PL	SM	PC	PE	PS	PI
MPL-rich ingredient	25.9 ± 0.2	27.3 ± 1.5	24.8 ± 0.1	11.9 ± 0.9	9.8 ± 0.4

Each value represents the mean ± SEM of the values for n=2 batches of extracted MPL.

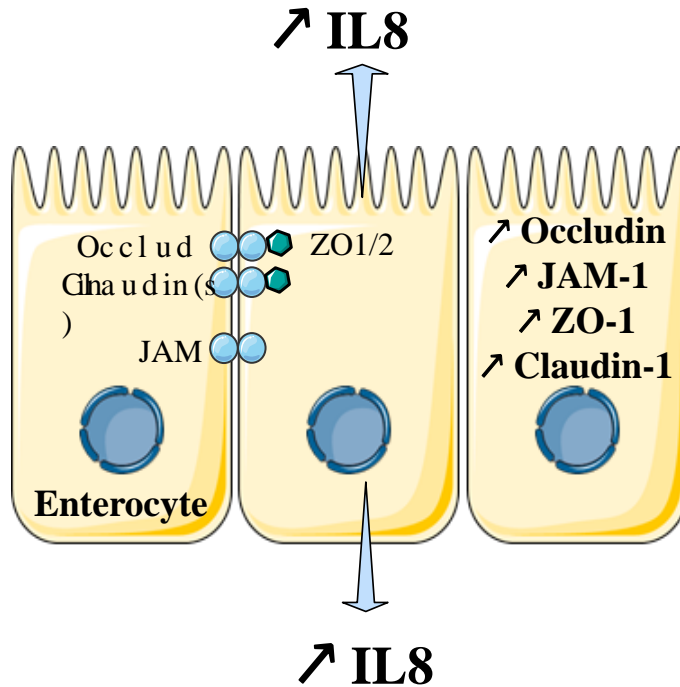
¹ MPL, milk polar lipid-rich ingredient; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, polar lipid; PS, phosphatidylserine; SM, sphingomyelin.

“Mixed micelles”

mimicking postprandial lipid structures in gut lumen



No effect



↗ **IL8**



↗ **Occludin**
↗ **JAM-1**
↗ **ZO-1**
↗ **Claudin-1**

↗ **IL8**

Up-regulation

Interleukin-8

