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

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

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22

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

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 chemoattactant (KC) (CXCL1) and macrophage

97 inflammatory protein-2α (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 puschased 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^{TM}; 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% CO_2 atmosphere at 37°C. Cells were seeded at a density

131   of 2.5 x 10^5 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^{TM} glass

203   antifade mountant with NucBlue^{TM} (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 µL of 25 mg/mL of MSM (corresponding to 5 mg)

222   -   **MSM 2**: 200 µL of 50 mg/mL of MSM (corresponding to 10 mg)

223   -   **MPL 1:** 200 µL of 96.2 mg/mL of MPL (corresponding to 5 mg of SM)

224   -   **MPL 2**: 200 µL 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% O_2 until

230   unconscious and anaesthesia was maintained with 2% isoflurane in 100% O_2). 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-1β, 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 Reageant (Sigma, Saint-Quentin-Fallavier, France) and suspended in RNase-free water.

246 RNA concentration was measured with Multiskan[TM] 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[TM] RT reagent kit (Ozyme, Saint Quentin en Yvellines, 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 ± 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) <mark>(an asterisk represents a significant difference</mark>

269   <mark>with control).</mark>

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_{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 0.4mM 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_{source}<0.01) and for the mRNA expression (Fig. 1E, P_{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-1β 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, $P_{source}<0.05$). *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-1β, 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, $P_{source}<0.05$ in the duodenum and jejunum) and *Zo-1* (Fig S6G, I,

357    $P_{source}<0.05$ 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 (IFNγ) [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-1β 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.

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.

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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 ± 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 ± 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

<mark>MSM corresponds to 0.16 mg/mL.</mark> 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 ± 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 adhestion 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). <mark>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).</mark> 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 ± SEM. <mark>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).</mark> 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.
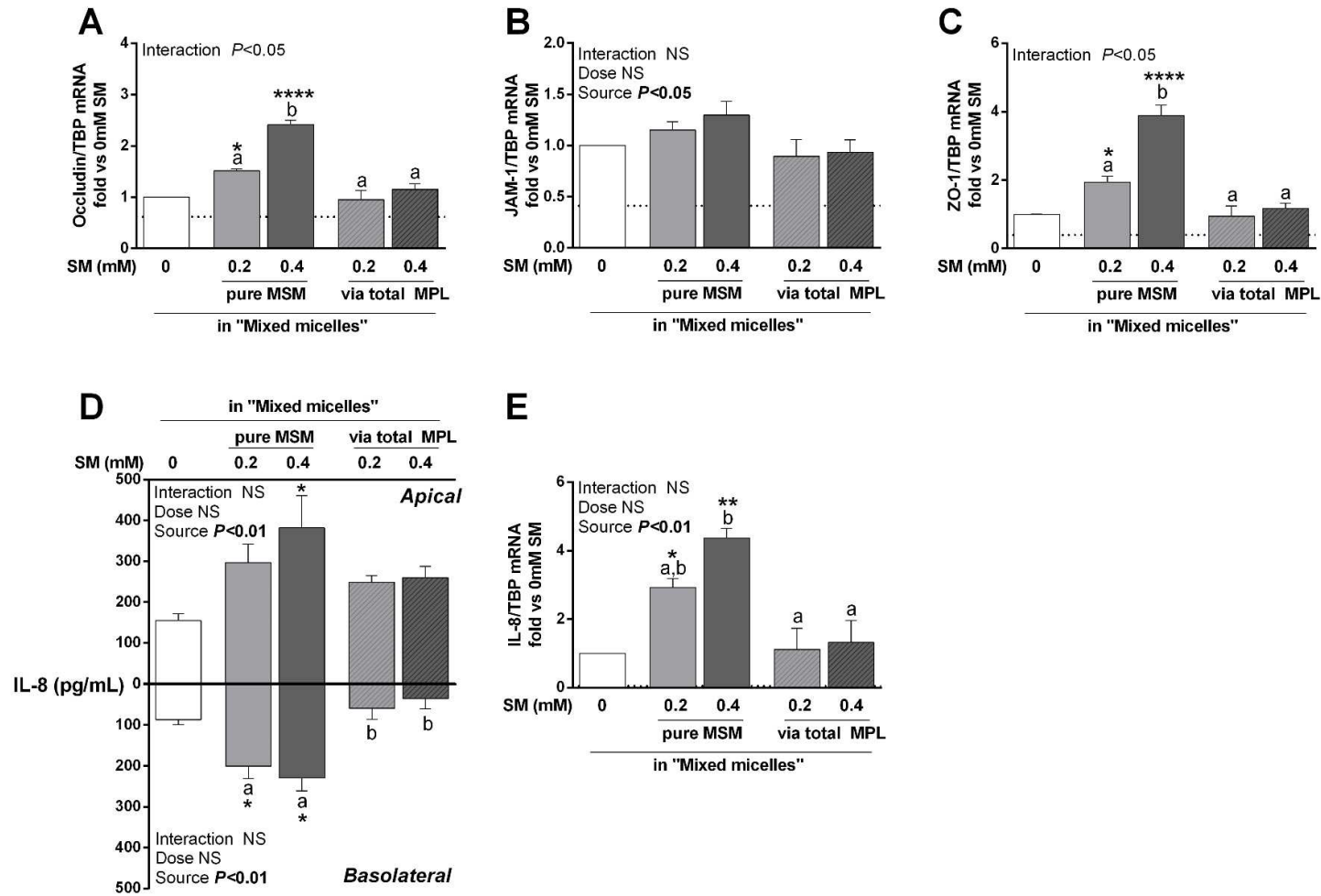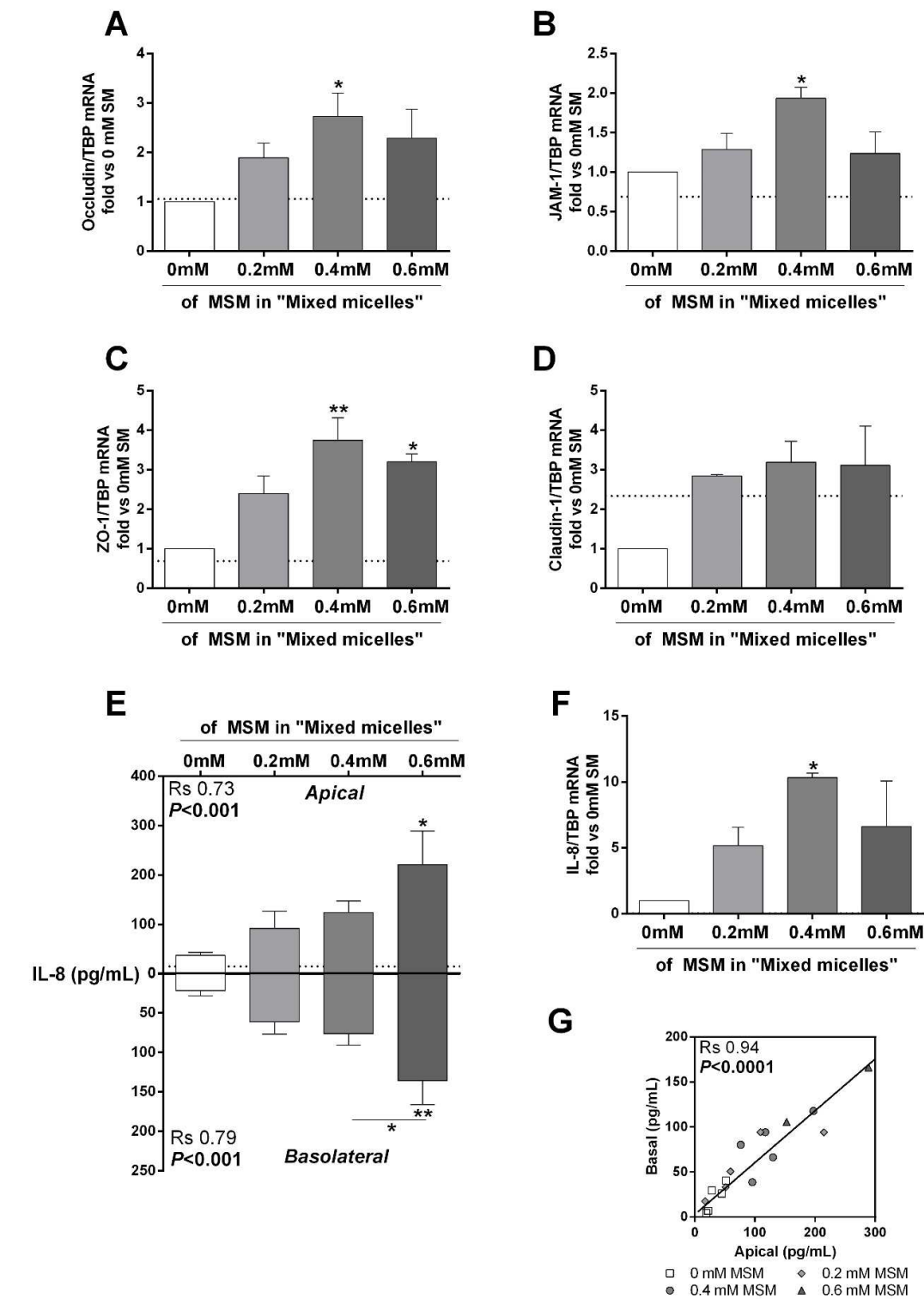
Figure 1.

Figure 2.

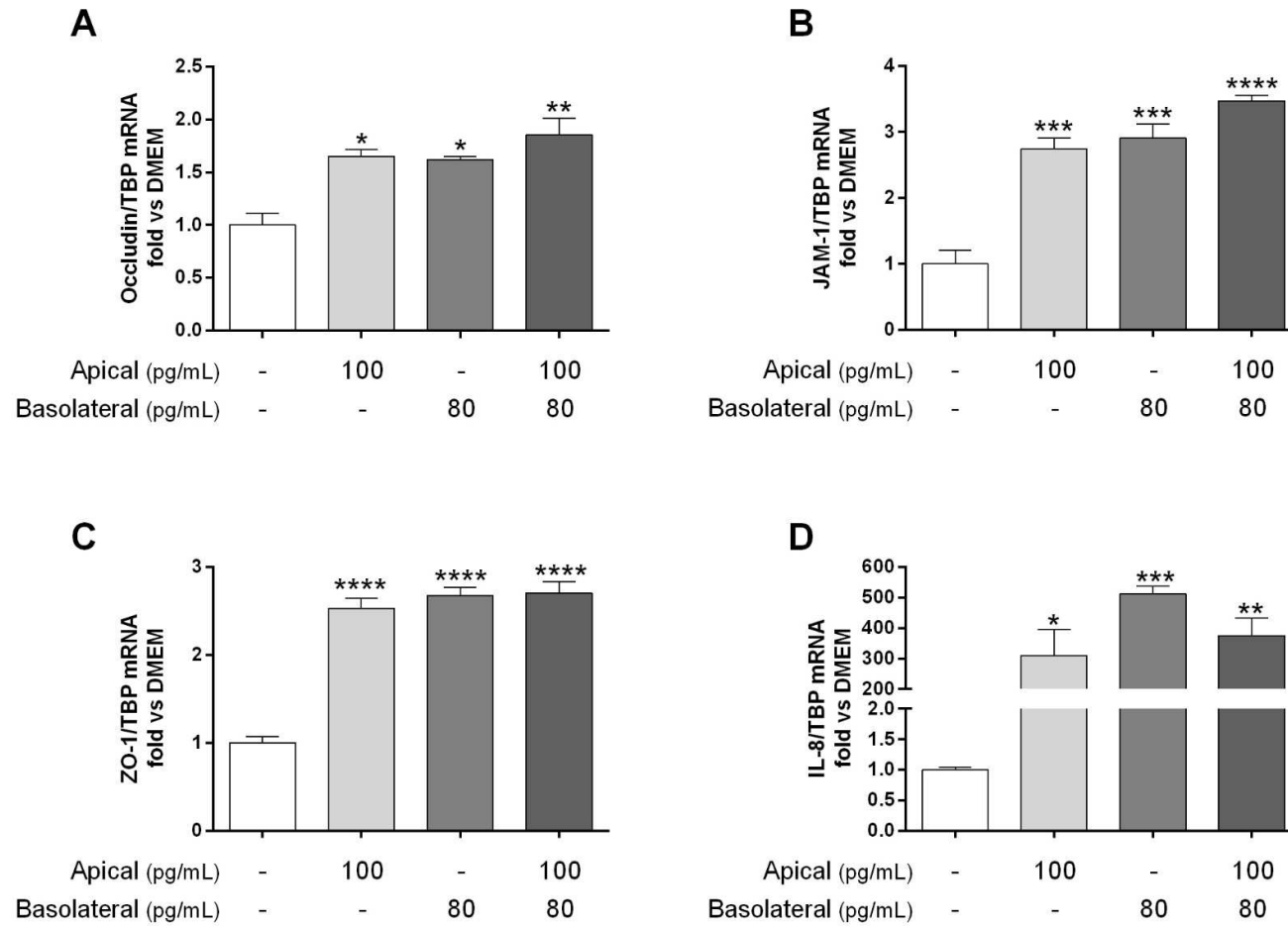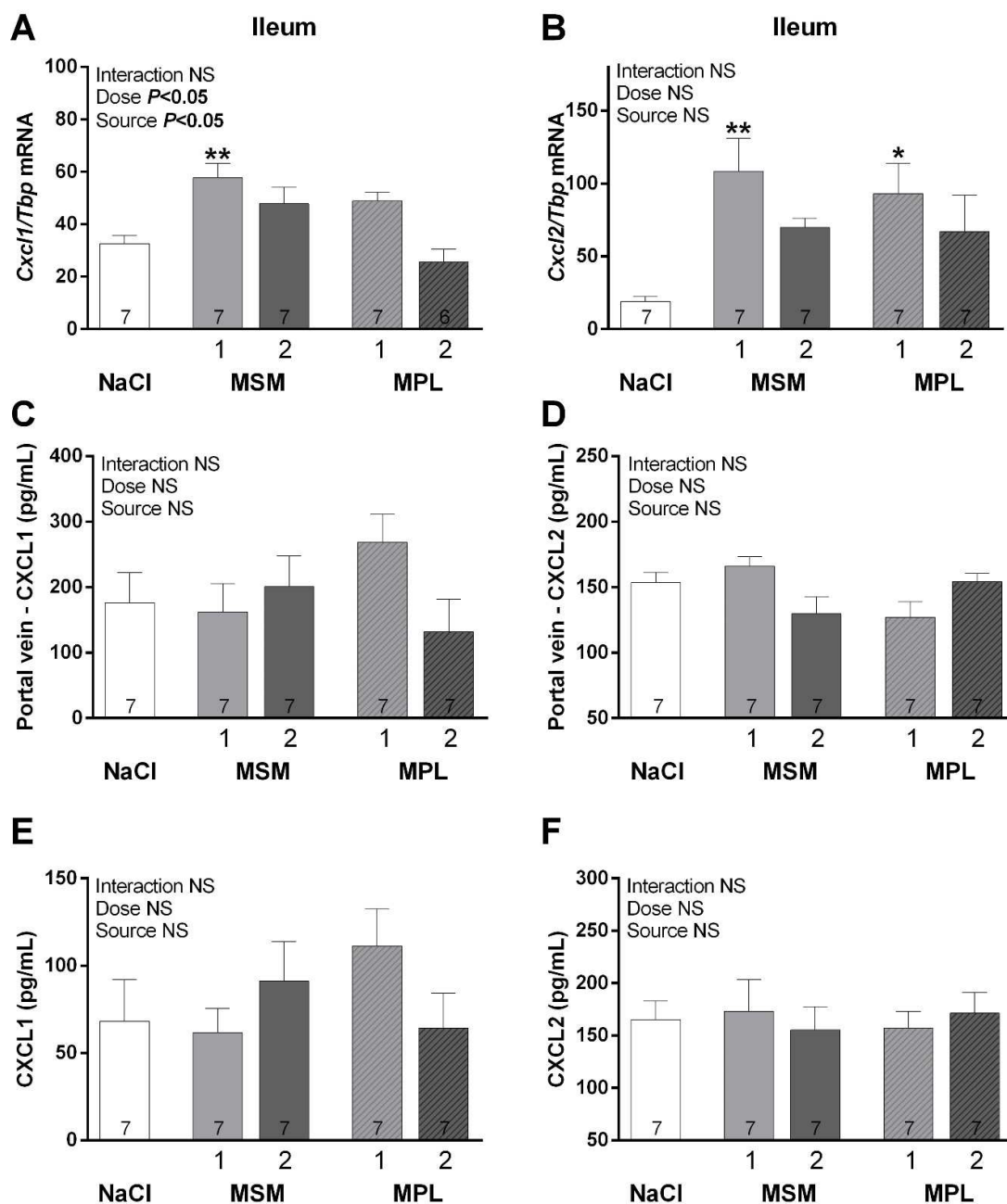**Figure 3.**

Figure 4.

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

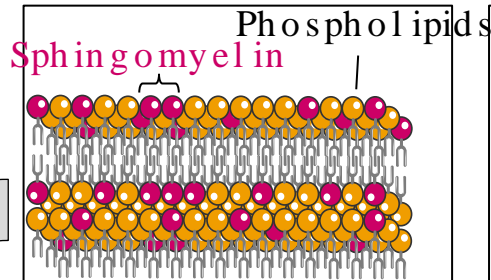| PL classes, % total PL	SM	PC	PE	PS	PI
MPL-rich ingredient	25.9 ± 0.2	27.3 ± 1.5	24.8 ± 0.1	11.9 ± 0.9	9.8 ± 0.4

Each value represents the mean $\pm$ 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;
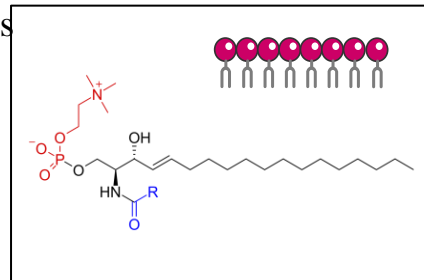
SM, sphingomyelin.

"**Mixed micelles**"
mimicking postprandial lipid structures in gut lumen
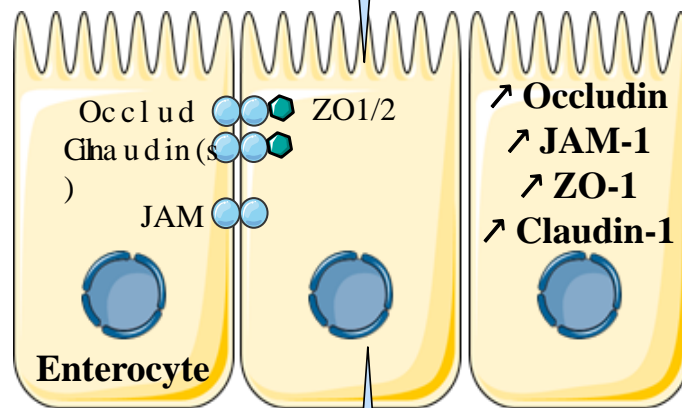
Total milk polar lipids

Pure Sphingomyelin

Sphingomyelin

Phospholipids

Interleukin-8

↗ **IL8**

No effect

Occludin
Claudin(s)

ZO1/2

JAM

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

Up-regulation

Enterocyte

↗ **IL8**