Immunomodulatory effects of long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) on porcine monocytes (CD14+) immune response *in vitro*

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ABSTRACT

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are omega-3 long-chain polyunsaturated fatty acids (n-3 PUFA) found mostly in fish oil. They have been commonly used as dietary integrators in human and animal nutrition, modulating the immune system, mostly by exerting anti-inflammatory activities as demonstrated by *in vivo* and *in vitro* studies. The precise mechanisms of action at the background of EPA and DHA immunomodulatory activity are still not fully elucidated. Moreover, no information

on their effects on porcine monocytes immune response is available yet. To cover this gap, the study aimed to evaluate DHA and EPA's *in vit*ro impact on porcine monocytes (CD14+) defensive functions. Briefly, monocytes were isolated from the blood of twenty-six healthy pigs, using a magnetic-activated cell sorting technique (MACS). Monocytes were first treated with increasing concentrations of DHA and EPA (25, 50, 100 and 200 µM) and apoptosis and viability were measured to assess potential cytotoxic effects. Once determined EPA and DHA subtoxic working concentrations (25, 50 and 100 µM), their effects on chemotaxis, phagocytosis and total, intracellular and extracellular reactive oxygen species (ROS) production were evaluated. DHA and EPA only decreased porcine monocytes viability at the highest concentration (200 µM), but their apoptosis was unaffected. DHA (100 µM) decreased the cells' chemotaxis, while EPA (25 µM) increased their intracellular ROS production after 60 minutes under non-inflammatory or resting conditions and at 90 minutes under pro-inflammatory conditions (PMA challenge). EPA (50 µM) decreased monocytes' intracellular ROS levels only under resting conditions at 30 minutes. No effects were observed on porcine monocytes phagocytic capacity. In conclusion, this study demonstrates that DHA and EPA can exert differential *in vitro* immunomodulatory effects in pigs, by dampening monocytes chemotaxis and potentiating their oxidative burst, respectively. Thus, our results suggest these n-3 PUFA might exert both anti-inflammatory and/or immune-enhancing effects in pigs.

Keywords*:* n-3 PUFA; Pig; Monocyte; Chemotaxis; ROS production; Anti-inflammatory

Abbreviations

PUFA, polyunsaturated fatty acids; MACS, magnetic-activated cell sorting; FA, fatty acids; ALA, alpha-linolenic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic

51 acid; DHA, docosahexaenoic acid; ARA, arachidonic acid; GPR120, G protein-coupled
52 receptor 120; PPARγ, peroxisome proliferator-activated receptor γ; NFκB, nuclear factor
53 κB; SPM, specialized pro-resolving lipid mediators; MTT, 3-(4,5- dimethyl thiazol -2-
54 yl)-2,5-diphenyl tetrazolium bromide; ROS; reactive oxygen species; ZAS, zymosan
55 activated serum; H_2DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; HRP,
56 horseradish peroxidase; DPI, diphenyleneiodonium chloride; O_2^-, superoxide anion;
57 H_2O_2, hydrogen peroxide;
58

59 ## 1. Introduction

60 Omega-3 polyunsaturated fatty acids (n-3 PUFA) are fatty acids (FA) that naturally
61 contain more than one double bond in their structure (De Caterina, 2011). They are
62 divided into 1) short chain n-3 PUFA: α-linolenic acid (ALA), and 2) the longer-chain
63 n3-PUFA: eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and
64 docosahexaenoic acid (DHA) (Calder, 2013). Long-chain n3-PUFA are mostly abundant
65 in, seaweeds and other fish oil supplements, being the diet their main source (Calder,
66 2015). EPA and DHA have been long used as dietary fatty acids in both human and animal
67 nutrition, as they have been demonstrated to have beneficial properties, improving their
68 antioxidant defense mechanisms, growth, fertility, health and immunity (Calder, 2007;
69 Fu et al., 2021; Lee et al., 2019).
70 As structural and functional components of the immune cells' membrane phospholipids,
71 EPA and DHA are known to mainly alter immune cell functions by changing the cells'
72 membrane FA composition after their incorporation, thus affecting the membrane's
73 fluidity, lipid raft formation, cell signaling and consequent gene expression (Gutiérrez et
74 al., 2019). Their immunomodulatory and anti-inflammatory actions can be also attributed
75 to other mechanisms such as: inhibition of arachidonic acid (ARA) metabolism, direct

76 interaction with the cell surface and intracellular receptors, G protein-coupled receptor
77 120 (GPR120) and the anti-inflammatory peroxisome proliferator-activated receptor γ
78 (PPARγ), respectively (Calder, 2013). The inhibition of ARA metabolism and direct
79 modulation of PPARγ signaling pathway have been associated with reduced production
80 of pro-inflammatory eicosanoids and expression inflammatory genes (Kong et al., 2010;
81 Li et al., 2005; Peterson et al., 1998). Moreover, EPA and DHA are known substrates for
82 anti-inflammatory and specialized pro-resolving lipid mediators (SPM) such as resolvins,
83 protectins, lipoxins and maresins that mediate the resolution of the inflammation (Chiang
84 and Serhan, 2020). The defensive actions of immune cells can be affected by EPA and
85 DHA *in vitro* and *in vivo,* including an increased secretion of anti-inflammatory
86 cytokines (IL-10 and TGFβ) and decreased secretion of pro-inflammatory ones (TNFα,
87 IL-1β and IL-6) in murine macrophages (Chang et al., 2015), the immunomodulation of
88 goat monocyte and neutrophil phagocytosis and ROS production (Lecchi et al., 2011;
89 Pisani et al., 2009). Finally, the increase of EPA and DHA in human diets also reduced
90 monocytes and neutrophils chemotaxis *ex vivo* (Schmidt et al., 1992).
91 In the past few years increased attention has been put to DHA and EPA supplementation
92 in pigs' diets, to exploit their beneficial properties, including immunomodulatory and
93 anti-inflammatory ones. The effects of n-3 PUFA coming from fish oil have been shown
94 to influence pigs' gut health and immunity, being of special importance for transition
95 periods such as postweaning, where the piglets are more susceptible to inflammation
96 (Lauridsen, 2020). A low dietary ω6:ω3 ratio (4:1) during gestation and lactation has
97 beneficial effects for weaning survival rate, weight gain, and ω3 enrichment in colostrum
98 and milk (Nguyen et al., 2020). Additionally, EPA and DHA caused *in vitro*
99 cytoprotective and proliferative effects on porcine enterocytes (IPEC-J2) submitted to
100 different biological and chemical stresses (LPS and H_2O_2) (Sundaram et al., 2020). These

101 findings confirmed the potential of EPA and DHA in maintaining pigs' gut health,
102 integrity and immunity, especially under pro-inflammatory conditions. Lastly, dietary
103 fish oil supplementation in weaned piglets also modulated the inflammatory responses of
104 porcine alveolar macrophages *ex vivo*, with a negative correlation between the n-3 PUFA
105 content and PGE_2, TNF-α and IL-8 concentrations, which lead to a decreased production
106 of pro-inflammatory cytokines and eicosanoids (Møller and Lauridsen, 2006).
107 Even though previous studies have already demonstrated the immunomodulatory effects
108 of EPA and DHA on pigs, they have mainly focused on assessing their effects on the
109 production of inflammatory cytokines, eicosanoids, and gut health and integrity. In
110 addition, the exact mechanisms underlying the effects of EPA and DHA on porcine
111 immune cells' response have not been yet fully elucidated, and to the best of our
112 knowledge, no studies of their *in vitro* impact on porcine monocytes - the main effectors
113 immune cells - are available. Therefore, in this study, we aimed at covering this gap by
114 investigating the *in vitro* effects that DHA and EPA may exert on porcine monocytes
115 defensive functions, including chemotaxis, phagocytosis and oxidative burst. Their
116 effects on the cells' viability and apoptosis were also evaluated.
117
118 **2. Materials and methods**
119
120 *2.1. Materials*
121 Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), EDTA 0.5 M,
122 red blood cell lysis buffer, and sterile-filtered Dulbecco's PBS without calcium and
123 magnesium (Sigma-Aldrich, St. Louis, MO, USA) were used for porcine PBMC
124 isolation. For monocytes purification CD14 MicroBeads, Large Size (LS) columns, 30-
125 mm pre-separation filters (Miltenyi-Biotech, Bergisch Gladbach, Germany) and 0.5%

126 BSA (Sigma-Aldrich) were used. Once isolated, the cells were resuspended in complete
127 medium RPMI 1640 with 25mM HEPES and L-glutamine complemented with 1%
128 nonessential amino acid solution 100× and 1% penicillin-streptomycin solution 100×, and
129 10% FBS (Sigma-Aldrich). DHA (cis-4,7,10,13,16,19-Docosahexaenoic acid sodium
130 salt) and EPA (cis-5,8,11,14,17-Eicosapentaenoic acid sodium salt) used for stimulating
131 the cells were purchased from Sigma-Aldrich. Zymosan A from *Saccharomyces*
132 *cerevisiae* (Sigma-Aldrich), BioParticles® *E. coli* Fluorescent Particles (Invitrogen) were
133 used for chemotaxis and phagocytosis assay, respectively. For the ROS production
134 assays, 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), luminol, 4-
135 Aminophthalhydrazide (isoluminol), horseradish peroxidase (HRP),
136 diphenyleneiodonium chloride (DPI) and PMA were purchased from Sigma-Aldrich.
137

138 *2.2.PUFA preparation*

139 DHA (14.3 mM) and EPA (15.4 mM) stock solutions were prepared by adding 1 mL of
140 endotoxin-free water (Sigma-Aldrich). The stock solutions were then filtered with 0.22
141 µm filters (Millipore Corporation, Billerica MA, WA, USA), aliquoted and stored in the
142 dark at -80 °C until use. Immediately before their use, EPA and DHA working dilutions
143 were prepared with complete medium. Increasing concentrations of DHA and EPA (0,
144 25, 50, 100 and 200 μM) were first used for determining their working concentration, as
145 previously described for caprine monocytes (Lecchi et al., 2011). The subtoxic
146 concentrations of 25, 50 and 100 μM were selected to perform the further experiments.
147

148 *2.3. Purification of porcine monocytes (CD14+) from blood*

149 Peripheral blood (100 mL) from twenty-six 60-100 kg healthy pigs (TOPIGS) was
150 collected during routine slaughtering procedures in sterile flasks containing 0.2% of

151 EDTA as an anticoagulant. PBMC were isolated first through Ficoll density gradient
152 centrifugation, as described for bovine blood with some minor modifications (Ceciliani
153 et al., 2007). Briefly, blood was first centrifuged at 1260 g for 30 min at 18 °C to collect
154 the buffy coat. The collected buffy coat was diluted 1:5 in cold sterile-filtered PBS
155 without Ca^{2+} and Mg^{2+} + 2 mM EDTA and carefully layered onto 3 mL of Ficoll-Paque
156 Plus (1.077g/mL). A second centrifugation step was performed at 1700 g (without brakes)
157 for 30 min at 4 °C to obtain the PBMC band. The PBMC were collected at the interface,
158 washed with cold sterile PBS without Ca^{2+} and Mg^{2+} + 2 mM EDTA and centrifuged at
159 500 g for 7 min at 4 °C. To remove the red blood cells, Red Blood Cell Lysis buffer was
160 added to the PBMC and then incubated for 3 min at room temperature. Washes with cold
161 sterile PBS + 2 mM EDTA were performed to remove contaminant platelets. Monocytes
162 (CD14+) were further purified from PBMC through magnetic-activated cell sorting
163 technique (MACS), as previously described for bovine samples (Ávila et al., 2020),
164 following the manufacturer's instructions. Monocytes were counted and their viability
165 assessed through Trypan blue exclusion (>90%), using an automatic cell counter
166 (TC20^{TM}, BioRad). Finally, monocytes were resuspended in complete medium at the
167 desired concentration for each assay.
168
169 *2.4. Viability assay*
170 To assess potential cytotoxic effects of DHA and EPA and to select the subtoxic working
171 concentrations, the cell proliferation kit I (MTT) (Roche Diagnostics GmbH, Mannheim,
172 Germany) was used, following the manufacturer's instructions and as previously
173 described with bovine monocytes (Catozzi et al., 2020). Briefly, monocytes ($1x10^5$) from
174 7 different animals were seeded in duplicates in clear sterile 96-well plates (Becton
175 Dickinson and Company, Franklin Lakes, NJ, USA) and incubated with increasing

176 concentrations of DHA and EPA (25, 50, 100 and 200 μM) or with only medium (0 μM)
177 as a control for 22 h at 39 °C + 5% CO_2. After the incubation time, the MTT labelling
178 reagent (10 μL) was added and the cells were incubated for 4 hours at 39°C + 5% CO_2.
179 To solubilize the produced formazan crystals, 100 μL of solubilisation buffer was added
180 and the plates were incubated overnight at 39 °C + 5% CO_2. The absorbance was
181 measured with a LabSystems Multiskan plate reader spectrophotometer (LabX, Midland,
182 Canada) at 550 nm.
183
184 *2.5.Apoptosis assay*
185 To evaluate the effects of DHA and EPA on porcine monocytes apoptosis, the enzymatic
186 activity of Caspase-3/7 was measured, as previously described for bovine monocytes with
187 minor modifications (Ceciliani et al., 2007). Briefly, $5x10^4$ monocytes (12.5 μL) from 6
188 animals were seeded in duplicates in sterile black 384-well plates (Corning Inc., Costar,
189 Kennebunk, ME, USA) and incubated with increasing concentrations (25, 50, 100 and
190 200 μM) of EPA and DHA (12.5 μL) or with only medium (0 μM) as a control for 22
191 hours at 39 °C + 5% CO_2. After the incubation period, the cells' apoptosis was measured
192 using the Apo-ONE® Homogeneous Caspase-3/7 kit (Promega, Madison, WI, USA),
193 following the manufacturer's instructions. The fluorescence was then measured every 30
194 minutes for 2 hours with the Fluoroscan Ascent (Thermo Fisher Scientific, Vantaa,
195 Finland) at 485/538 nm (absorbance/emission).
196
197 *2.6.Chemotaxis assay*
198 Monocytes chemotaxis towards zymosan activated serum (ZAS) was measured as
199 previously reported (Ávila et al., 2020; Lecchi et al., 2008), with some minor changes.
200 Firstly, $1x10^5$ monocytes (50 μL) from 6 animals were seeded in duplicates onto the semi-

201 permeable membrane (8 µm pore size) of the upper chamber of a sterile 24-well Transwell
202 migration plate (Corning Inc., Costar). The cells were then pre-treated, in the absence of
203 ZAS, with 100 µM of DHA or EPA (50 µL in the upper chamber and 650 µL in the lower
204 chamber) or only migration medium (RMPI-1640 with 1% of heat-inactivated FBS) as a
205 control for 22 hours at 39 °C + 5% CO_2. After the incubation period, the medium was
206 removed from both chambers and again 100 µM of EPA and DHA or migration medium
207 were added to both chambers. To measure the cells' chemotaxis, 3 mg/mL of ZAS were
208 added only to the lower chamber of the plate in both, the PUFA and medium treated cells
209 (positive control), and cells were incubated further for 2 hours at 39 °C + 5% CO_2. Cells
210 incubated only with migration medium, but without ZAS were considered as the negative
211 control. Finally, the non-migrated cells were removed from the upper membrane, and
212 those that migrated were stained using Diff Quick Staining (Sigma-Aldrich) and counted
213 using an inverted microscope at 40x. ZAS was prepared using porcine serum, as
214 previously described for bovine monocytes (Lecchi et al., 2008).
215
216 *2.7.Phagocytosis assay*
217 Monocytes' phagocytic activity was determined as previously described (Lecchi et al.,
218 2011). Briefly, a total of $3x10^5$ monocytes (100 µl) from 7 different animals were seeded
219 in duplicates in sterile 96-well plates (Becton Dickinson and Company). They were
220 incubated with increasing concentrations (25, 50 and 100 µM) of EPA and DHA (100 µl),
221 respectively or with only medium (0 µM) as a control for 22 hours at 39 °C and 5% CO_2.
222 After the incubation period, fluorescein-labelled *Escherichia coli bioparticles* (K-12
223 strain*)* were first opsonized with 20% non-decomplemented pig serum at 39 °C for 30
224 minutes. Cells were then washed with sterile PBS and 100 µl of opsonized
225 bioparticles/cell (45 bioparticles/cell) were added to the wells and cells were incubated

226 for 2 hours at 39 °C and 5% CO_2. Non-internalized bioparticles were then removed by
227 washing the cells with PBS and, their fluorescence was further quenched by incubating
228 the monocytes with 50 µl of 0.4% trypan blue for 1 minute at room temperature. Trypan
229 blue was then washed, and PBS (100 µl) was added to all the wells. Finally, the
230 fluorescence was measured using the microplate reader Fluoroscan Ascent FL (Thermo
231 Fisher Scientific) at 485/538 nm (absorbance/emission).

232

233 *2.8. Total, intracellular and extracellular ROS production assay*

234 The production of intracellular, total and extracellular - superoxide anion (O_2-) ROS were
235 determined under both, resting and pro-inflammatory conditions (PMA challenge), with
236 H_2DCFDA, luminol and isoluminol in the presence of HRP, respectively as previously
237 described for bovine polymorphonuclear cells (PMN) with some minor modifications
238 (Grob et al., 2020; Rinaldi et al., 2007; Wang et al., 2020). Briefly, a total of $1x10^5$
239 monocytes (100 µL) from 6 animals was seeded in complete medium without phenol red
240 in duplicates in sterile black (Corning Inc., Costar) or white 96-well plates (Nunclon Delta
241 Surface, Thermo Fisher Scientific) for intracellular; and total and extracellular ROS
242 assays, respectively. In addition, for all the ROS assays, cells were then co-incubated with
243 increasing concentrations of DHA or EPA (25, 50 and 100 µM) or only medium as control
244 (0 µM) for 22 h at 39 °C + 5% CO_2.

245 Specifically, for the intracellular ROS evaluation, after the incubation period, cells were
246 washed twice with PBS at room temperature to remove the FBS, as it can interfere with
247 the activation of the fluorescent ROS indicator probe, H_2DCFDA. To load the fluorescent
248 probe into the cells, 200 µL of H_2DCFDA (10 µM final concentration) or PBS alone
249 (negative control) were added and cells were incubated for 20 min at 39 °C + 5% CO_2.
250 The loading buffer was then removed and 200 µL of medium without phenol red and FBS

251 or 200 μL of PMA (2.5 μg/mL of final concentration) were added to the cells to recreate
252 the non-inflammatory (resting) and pro-inflammatory conditions, respectively.
253 Fluorescence intensity was measured immediately (0 min) and then every 30 min for 2 h,
254 using a fluorescence plate reader Fluoroscan Ascent at 485/538 nm
255 (absorbance/emission).
256 For the total ROS and extracellular O_2^- production determination, 50 μL of luminol (500
257 μM final concentration) or isoluminol (100 μM final concentration) + 4 U/mL HRP were
258 added to the cells to reach a final volume of 200 μL, respectively. To mimic the pro-
259 inflammatory conditions, PMA (2.5 μg/mL final concentration; Sigma-Aldrich) was also
260 added to the cells. The effect of the ROS inhibitor, DPI – a NADPH oxidase inhibitor -
261 on monocytes total and extracellular ROS generation was also assessed, so monocytes
262 treated with only medium (no PUFA) were first treated with 8 μM of DPI for 10 min at
263 39 °C + 5% CO_2 and then luminol and isoluminol + HRP, with or without PMA, were
264 added as mentioned above. The chemiluminescence was then immediately measured (0
265 min) and then every 30 min for 2 h using a plate reader luminometer (Glomax 96
266 Microplate Luminometer, Promega). Data for total and extracellular ROS assays are
267 presented as relative chemiluminescence units (RLU).
268

269 *2.9.Statistical analyses*

270 Statistical analyses were performed in GraphPad Prism 9.1.2 for Mac OS X, GraphPad
271 Software (San Diego, California, USA). Data normality was assessed by applying the
272 Shapiro-Wilk test. For normally distributed data, a repeated measures one-way ANOVA
273 followed by Tukey's multiple comparison tests was used for all the assays; while for not
274 normally distributed data in the ROS production assays a Friedman test followed by
275 Dunn's multiple comparisons test was applied. Specifically, for ROS production assays

276 a repeated measures one-way ANOVA or Friedman test was applied to assess exclusively
277 the effect of the different treatments (DHA and EPA concentrations and control) within
278 each time point (0, 30, 60, 90 and 120 min). Statistical differences were accepted at $P \leq$
279 0.05.

280

281 **3. Results**

282

283 *3.1. DHA and EPA affect porcine monocytes' viability at high concentrations*

284 As the first step of this study, a preliminary experiment was performed to determine the
285 subtoxic working concentrations of EPA and DHA. Only the highest concentration (200
286 µM) of DHA and EPA ($P = 0.01$) had cytotoxic effects on porcine monocytes, as a
287 decrease in the cells' viability was observed when compared to the control (Fig. 1A, B).
288 Porcine monocytes viability was also decreased at 200 µM of DHA when compared to
289 100 µM DHA, 50 µM DHA, and 25 µM DHA ($P = 0.01$); and 200 µM of EPA when
290 compared to EPA 100 µM EPA, 50 µM EPA ($P = 0.01$), and 25 µM EPA ($P = 0.02$).
291 Despite a decrease in the cell viability was observed at 200 µM of DHA and EPA, no
292 effects on the apoptosis of porcine monocytes were observed when treated with the
293 increasing concentrations of DHA (Fig. 1C) nor EPA (Fig. 1D).

294

295 *3.2.DHA modulates porcine monocytes' chemotaxis*

296 Porcine monocytes' chemotactic activity towards the chemoattractant, ZAS, was
297 measured after pre-treating the cells with either 100 µM of DHA or EPA or medium
298 (positive control) for 22 h. DHA suppressed ($P = 0.05$) the chemotaxis of porcine
299 monocytes, as the number of migrated cells was lower when compared to the positive
300 control without PUFA (Fig. 2A). No effects were observed with 100 µM of EPA (Fig.

301 2B). Finally, as expected positive control cells also presented a higher number of migrated
302 cells (P = 0.01) when compared to those not activated with ZAS (negative control),
303 confirming the chemoattractant activity.
304

305 *3.3.DHA and EPA don't have any effects on porcine monocytes' phagocytic capacity*

306 To further study EPA and DHA's effects on porcine monocytes defensive response,
307 porcine monocytes phagocytic capacity was measured, using the fluorescein-labelled *E.*
308 *coli* bioparticles internalization assay. However, increasing concentrations (25, 50 and
309 100 µM) of both DHA (Fig. 3A) and EPA (Fig. 3B) did not modulate porcine monocytes
310 phagocytosis when compared to the control (0 µM).
311

312 *3.4.EPA increases porcine monocytes' intracellular ROS production*

313 No effects with DHA on the cells' intracellular ROS production were observed under
314 resting conditions (non-inflammatory) nor pro-inflammatory conditions (Fig. 4A, B)
315 within each timepoint. On the contrary, cells treated with 25 µM of EPA showed an
316 increase (P = 0.05) in their intracellular ROS production at 60, 90 and 120 minutes, under
317 resting conditions, as compared to control (Fig. 4C), while 50 µM of EPA caused a
318 decrease (P = 0.03) in the cells' ROS production at 30 minutes under resting conditions.
319 Lastly, under pro-inflammatory conditions, only cells treated with 25 µM of EPA had an
320 increase in their intracellular ROS production, but only at 90 minutes (P = 0.01), when
321 compared to the control (Fig. 4D). Even though there is an increasing trend in the ROS
322 production when compared to the control, no significant effects were observed with 25
323 µM of EPA at 120 min (P = 0.06).
324

325 *3.5.Total and extracellular ROS Production is not affected by DHA and EPA*

326 Firstly, under resting conditions, porcine monocytes did not produce detectable total ROS
327 (Fig. 5A, C) when measured with the luminol chemiluminescence assay, as only negative
328 values were obtained and similar to those observed in the cells treated with the ROS
329 inhibitor (DPI) and the negative control (cells without luminol). In a same manner, the
330 extracellular ROS (Fig. 6A, C), which were measured with the isoluminol + HRP
331 chemiluminescence assay, were also not detectable during the first 60 minutes, but started
332 to increase in the cells treated with 100 μM of DHA and 50, 25 μM of EPA and control
333 (0 μM) at 90 and 120 min. EPA at 100 μM slightly increased the ROS production to
334 detectable levels at 90 min, but it decreased again at 120 min. Under pro-inflammatory
335 conditions, all cells treated with the increasing concentrations with DHA, EPA or without
336 PUFA (control) produced detectable total (Fig. 5B, D) and extracellular ROS (Fig. 6B,
337 D) after being challenged with PMA, while for those treated with the negative control or
338 DPI (ROS inhibitor) remained undetectable. Finally, none of the increasing
339 concentrations (25, 50 and 100 μM) of DHA or EPA modulated porcine monocytes total
340 (Fig. 5) nor extracellular ROS production (Fig. 6), neither under resting nor pro-
341 inflammatory conditions when compared to control (0 μM).

342

343 **4. Discussion**

344

345 The immunomodulatory and anti-inflammatory effects of the n-3 PUFA, EPA and DHA,
346 have been widely reported in both human and animal studies (Al-Khalaifah, 2020; Calder,
347 2017). However, no information on their direct *in vitro* effects on porcine monocytes
348 defensive functions is available yet. We present in this study for the first time the capacity
349 of DHA and EPA in modulating two main porcine monocytes' inflammatory functions,
350 namely chemotaxis and intracellular ROS production. Our main findings were that the

351 cells' chemotaxis was suppressed by DHA, and their intracellular ROS production was
352 increased by EPA under both, resting and pro-inflammatory conditions.
353 In the first part of the study, we observed that DHA and EPA decreased porcine
354 monocytes' viability at the highest concentration (200 µM). Interestingly, despite the
355 clear reduction in the viability of the cells - determined by measuring their metabolic
356 activity - at this concentration, the apoptosis of the cells remained unchanged. These
357 results suggest, that EPA and DHA cytotoxic effects might not be induced through
358 apoptosis, but possibly by other cell death mechanisms such as necrosis. Similarly, in a
359 previous study it was observed that the n-3 PUFA ALA also caused an increase in the
360 percentage of dead prostate cancer cells, but did not affect their apoptosis (Eser O. et al.,
361 2013). In the same study EPA did induce the cells' apoptosis, but the percentage of dead
362 cells still exceeded that of apoptotic cells, indicating that the cell death observed after
363 EPA treatment was not all attributed to apoptosis. Moreover, EPA and DHA have indeed
364 been shown to increase the necrosis in human cancer cell lines (Chiu and Wan, 1999;
365 Finstad et al., 1994); and both apoptosis and necrosis in macrophage cell line, but necrosis
366 in a greater extent (Martins de Lima et al., 2006). These results should be considered with
367 caution as they were reported in cancer and immune cell lines, which their response
368 greatly defer to that of healthy primary cells, but no information on primary immune cells
369 is available so far. However, our results are consistent with previous studies on caprine
370 monocytes, where also 200 µM of both PUFA decreased the viability of the cells (Lecchi
371 et al., 2011), and on human mononuclear cells in which concentrations higher than 150
372 µM caused the same effect (Jaudszus et al., 2013).
373 For all the following experiments, only the lower concentrations of DHA and EPA (25,
374 50 and 100 µM) were used, as no cytotoxic effects were observed in our and others

375 previous experiments *in vitro* at similar concentrations (Lecchi et al., 2011, 2013; Zhao
376 et al., 2005).
377 The effects of DHA and EPA were examined on three of the main defensive functions of
378 porcine monocytes, including chemotaxis, phagocytosis and ROS production. Only DHA
379 (100 µM) was able to reduce porcine monocytes chemotaxis towards ZAS. The ability of
380 DHA and EPA in suppressing immune cells migration has been widely documented in
381 other models such as in human neutrophils and monocytes (Schmidt et al., 1992), being
382 considered indeed as one of the key anti-inflammatory effects of these long-chain n-3
383 PUFA as reviewed elsewhere (Calder, 2017). Our study confirmed this effect in porcine
384 monocytes. The exact mechanisms by which DHA and EPA inhibit chemotaxis are not
385 fully clear yet, but this inhibition has been attributed to down-regulated expression of
386 receptors for chemoattractants, reduced production of some chemoattractants (e.g. LTB_4)
387 and down-regulated expression of adhesion molecule genes, via NFκB, PPAR-γ and
388 GPR120 (Calder, 2015). Additionally, SPM have been shown to inhibit the
389 transendothelial migration of human neutrophils (Serhan et al., 2000), which could be
390 caused by the reduction in actin polymerization (Krishnamoorthy et al., 2010), of
391 monocytes and macrophages as reviewed elsewhere (Balta et al., 2021). Lastly, reduction
392 in neutrophils' cell membrane fluidity after EPA incorporation, was also proposed to be
393 one of the reasons for the suppression of the cells' chemotaxis (Sipka et al., 1996).
394 In our experiment the porcine monocytes' phagocytic capacity was on the contrary not
395 affected neither by DHA nor EPA at any of the concentrations tested (25, 50 and 100
396 µM). These results differ from those observed in murine macrophages, and caprine
397 monocytes and neutrophils, where an increase in their capacity to engulf zymosan and
398 apoptotic cells (Chang et al., 2015), and *E.coli* were observed, respectively (Lecchi et al.,
399 2011; Pisani et al., 2009). However, the current information on the effects of DHA and

400 EPA on phagocytosis is limited, and contradictory, as other *in vivo* and *in vitro* studies
401 reported no effects at all (Rees et al., 2006), or a downregulating effect on phagocytosis
402 (Sipka et al., 1996).
403 In the last set of experiments, we assessed the effects of increasing concentrations of DHA
404 and EPA on porcine monocytes' overall capacity to generate ROS measuring the
405 intracellular, extracellular and total ROS levels, under both resting and pro-inflammatory
406 conditions (PMA-challenge). Our findings show that only EPA at the lowest
407 concentration (25 µM) was able to increase the production of intracellular ROS under
408 both resting and pro-inflammatory conditions at different time points. These findings
409 suggest that EPA might play an important role in enhancing the monocytes' immune
410 response and support the idea that PUFA should be considered a substantial source of
411 ROS (Schönfeld and Wojtczak, 2008). However, it should be also noted that EPA was
412 able to decrease the cells intracellular ROS production only under resting conditions and
413 at an intermediate concentration (50 µM) at 30 minutes. Our results agree with those
414 previously reported in rat neutrophils, where EPA caused a greater effect, by increasing
415 the *in vitro* production of intracellular (H_2O_2) and extracellular ROS (H_2O_2 and O_2^-) at
416 similar concentrations (12.5 - 150 µM), under resting and pro-inflammatory conditions
417 (Paschoal et al., 2013). Similarly, in LPS-stimulated murine macrophages EPA (10 and
418 100 µM) increased the total ROS production after the PMA challenge (Ambrozova et al.,
419 2010). Nevertheless, in our study, no impact on the extracellular O_2^- nor the total ROS
420 levels was detected, as determined with the isoluminol and luminol-dependent
421 chemiluminescence assays, respectively: this could be mainly due to the different
422 methods of ROS quantification used in the studies and the different cell types and
423 activation states. The lack of significant results observed is consistent with what was
424 found in goat monocytes, where DHA and EPA also didn't modulate their extracellular

425 O_2^- production (Lecchi et al., 2011). On the contrary, in caprine neutrophils only DHA
426 induced a down-regulation in extracellular O_2^- production *in vitro* (Pisani et al., 2009),
427 suggesting that these two n-3 PUFA exert differential immunomodulatory effects that
428 might also vary between species and cell types. Moreover, dual effects of DHA and EPA
429 in modulating extracellular O_2^- production in the mitochondria have already been reported
430 (Schönfeld and Wojtczak, 2008). The exact mechanisms underlying these dual effects of
431 EPA and DHA in immune cells ROS production are still not completely clarified, but it
432 is thought they could up-regulate ROS production via NADPH oxidase (Paschoal et al.,
433 2013), while a down-regulation could be due to a decreased transcription factor activation
434 (Gutiérrez et al., 2019).
435
436 **5. Conclusions**
437
438 In conclusion, the results of this study demonstrate for the first time that EPA and DHA
439 modulate different immune functions of porcine monocytes *in vitro*. Specifically, DHA
440 decreased porcine monocytes' chemotaxis, while EPA mainly increased their
441 intracellular ROS production under resting (non-inflammatory) and pro-inflammatory
442 conditions (PMA challenge). The results from the present study suggest that the long-
443 chain n-3 PUFA, DHA and EPA, may exert anti-inflammatory and/or immune-enhancing
444 effects on porcine monocytes, by downregulating their chemotactic capacity and
445 upregulating their oxidative burst, respectively. Therefore, our results further support the
446 hypothesis that EPA and DHA do not act only as unspecific immune repressors. EPA and
447 DHA's immunomodulatory properties make them attractive options to be used as dietary
448 supplements in pigs' diets. Thus, the information provided herein may be useful for the
449 design of targeted and balanced nutrition strategies. Specially, considering that high

450 concentrations of DHA and EPA had cytotoxic effects on porcine monocytes *in vitro*.
451 However, our study does not provide evidence on the exact molecular mechanisms
452 underlying such effects, and their elucidation using a system biology approach, including
453 transcriptomic and proteomic analyses, could help us to better understand EPA and
454 DHA's biological significance in *in vivo* systems.
455

456 **Declaration of Competing Interest**

457 The authors report no declarations of interest.
458

459 **Ethics statement**

460 All applicable international, national, and/or institutional guidelines for the care and use
461 of animals were followed. The procedures for the blood collection were carried out during
462 routine slaughtering procedures.
463

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473

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Figures

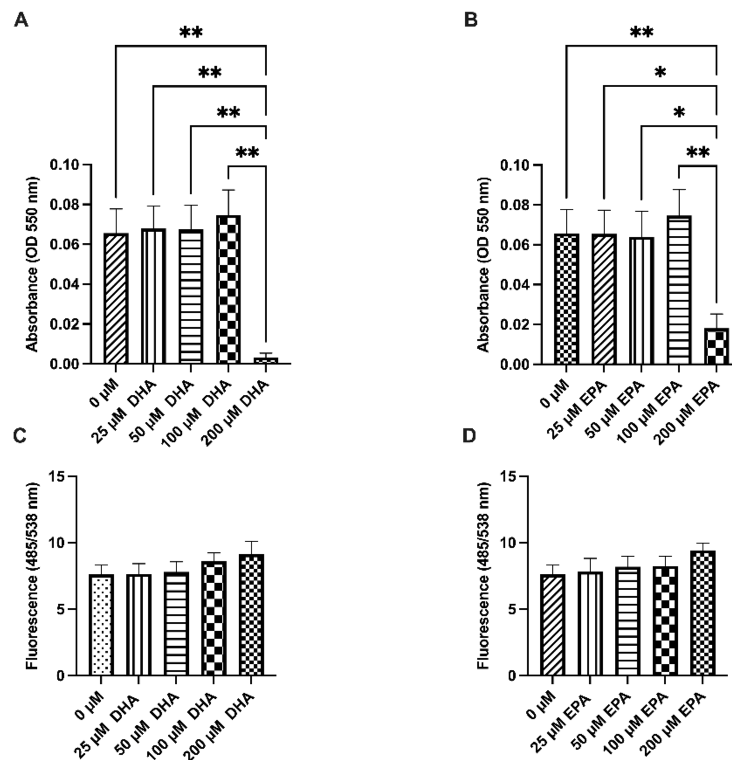

Fig. 1. *In vitro* effects of DHA and EPA on porcine monocytes' apoptosis and viability. Cells' viability after DHA (A) and EPA (B) treatment was measured. The effects of DHA (C) and EPA (D) on porcine monocytes apoptosis were also examined. The 2,5-diphenyl tetrazolium bromide (MTT) reduction by metabolically active cells and the caspase-3/7 enzymatic activity were measured for viability and apoptosis, respectively. The results are expressed as absorbance (OD 550 nm) for viability and fluorescence intensity (485/538 nm) for apoptosis. Data are means ± SEM of seven and six experiments for viability and apoptosis, respectively. OD = optical density. Significance was declared at P ≤0.05 (*) and P ≤ 0.01 (**).

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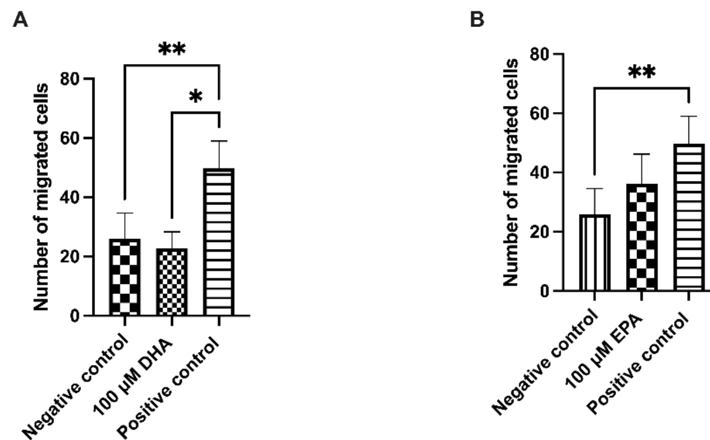

682 **Fig. 2.** *In vitro* impact of DHA and EPA, respectively on porcine monocytes chemotaxis.
683 Cells pre-treated for 22 h with 100 µM of DHA (A) or EPA (B), and only medium
684 (positive control) were activated with the chemoattractant Zymosan Activated Serum
685 (ZAS) in the presence or absence of DHA and EPA for 2 h, respectively. Cells pre-treated
686 with only medium (no PUFA) that were not activated with ZAS were considered as a
687 negative control. Data are means ± SEM of six experiments. Significance was declared
688 at P ≤0.05 (*) and P ≤ 0.01 (**).

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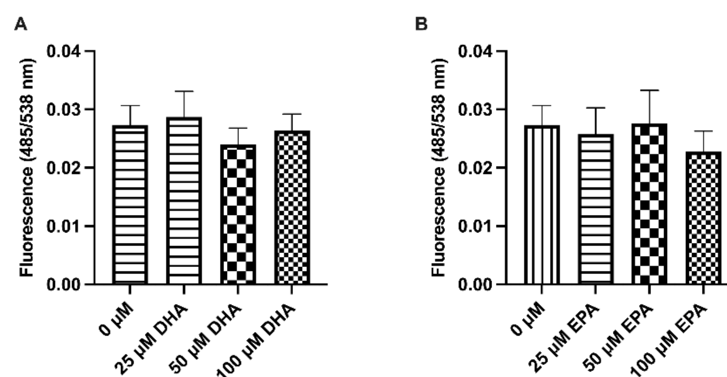

696 **Fig. 3.** Phagocytosis of fluorescein-labelled *Escherichia coli* bioparticles by porcine
697 monocytes treated with DHA and EPA. Cells were pre-treated with increasing
698 concentrations (25, 50 and 100 µM) of DHA (A) and EPA (B) or medium (0 µM) as a

699 control for 22 h. The results are expressed as fluorescence intensity (485/538 nm). Data
700 are means ± SEM of seven experiments.
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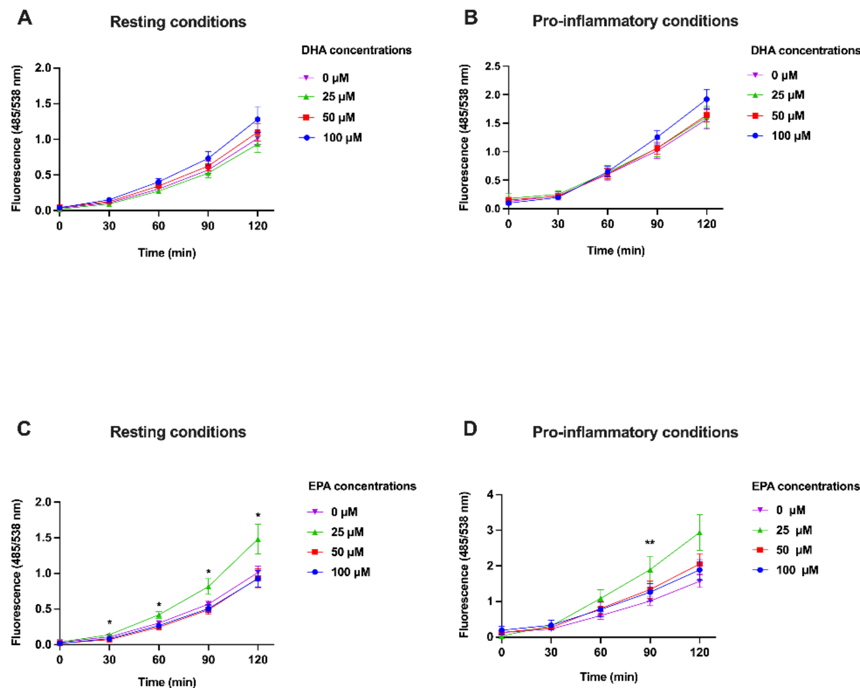

713 **Fig. 4.** *In vitro* effects of DHA and EPA on porcine monocytes' intracellular reactive
714 oxygen species (ROS) generation. Cells were first pre-treated with increasing
715 concentrations (25, 50 and 100 µM) of DHA and EPA or medium as control (0 µM) for
716 22 h. The intracellular ROS levels were then measured on the cells pre-treated with DHA
717 (panels A and B) or EPA (panels C and D) under both resting conditions or pro-
718 inflammatory conditions (PMA challenge), using the fluorescent ROS indicator,
719 H_2DCFDA. Fluorescence intensity (485/538 nm) was measured every 30 minutes for 2
720 h. Data are means ± SEM of six experiments. Significance was declared at $P \leq 0.05$ (*)
721 and $P \leq 0.01$ (**).
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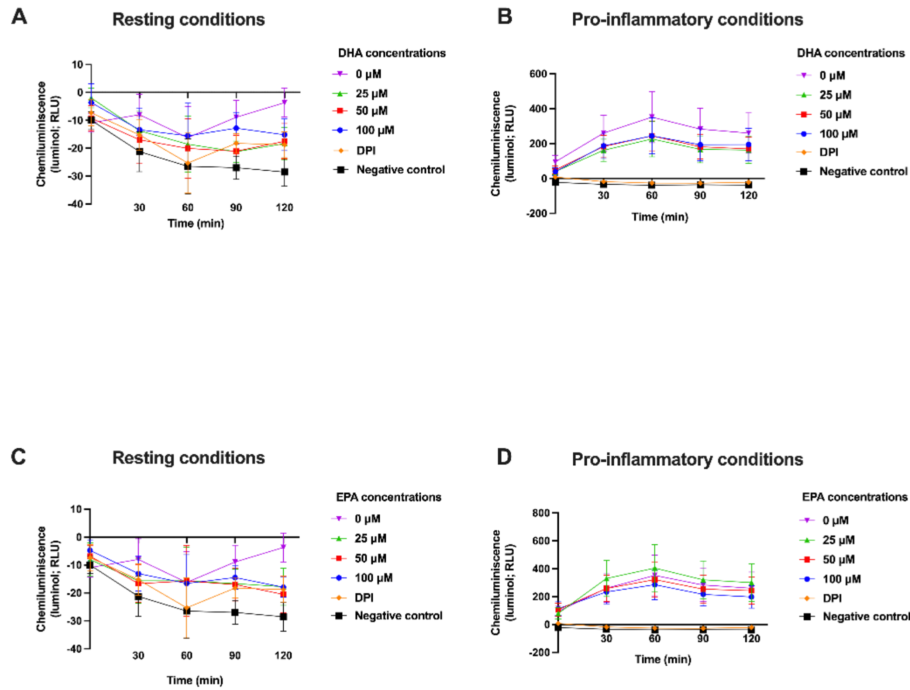

Fig. 5. Effects of DHA and EPA on *in vitro* porcine monocytes' total reactive oxygen species (ROS) generation. The total ROS levels were determined using luminol-dependent chemiluminescent assay, on the cells pre-treated with DHA (panels A and B) or EPA (panels C and D) under resting conditions or pro-inflammatory conditions (PMA challenge). Chemiluminescence was measured every 30 minutes for 2 h and the results are expressed as relative light units (RLU). Data are means ± SEM of six experiments.

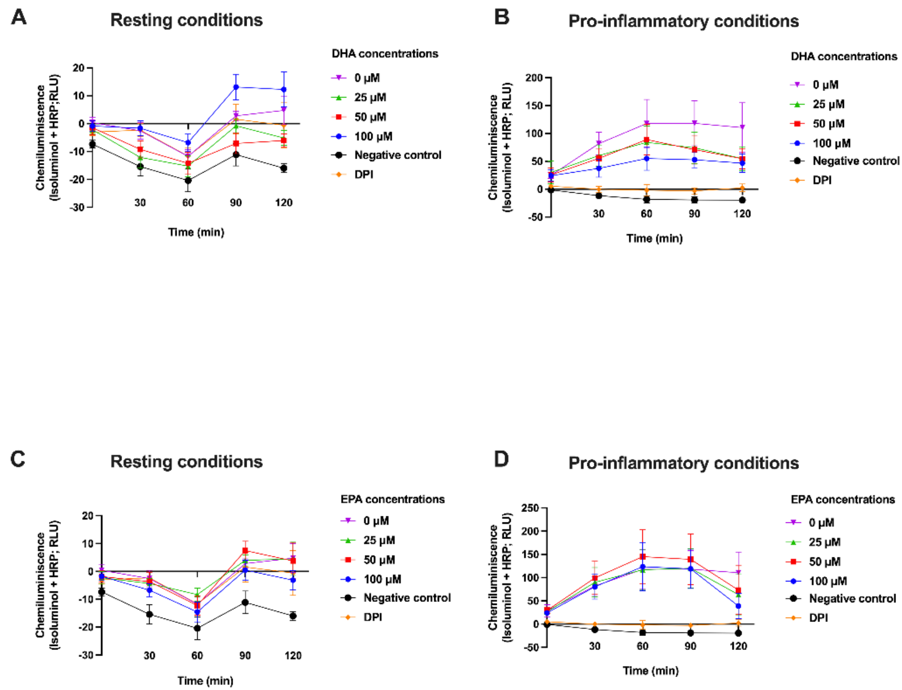

Fig. 6. Porcine monocytes' extracellular superoxide anion (O_2^-) production after DHA and EPA *in vitro* treatment. Cells were first pre-treated with increasing concentrations (25, 50 and 100 µM) of DHA and EPA or medium as control (0 µM) for 22 h. The extracellular ROS levels were then determined by adding isoluminol + horseradish peroxidase (HRP), on the cells pre-treated with DHA (panels A and B) or EPA (panels C and D) under resting conditions or pro-inflammatory conditions (PMA challenge). Chemiluminescence was measured every 30 minutes for 2 h and the results are expressed as relative light units (RLU). Data are means ± SEM of six experiments.