1 **Short Communication**

2 **Anti-inflammatory activity of citrus pectin on chicken monocytes' immune response**

3

4 *G. Ávila[a*], D. De Leonardis[a], G. Grilli[a], C. Lecchi[a], F. Ceciliani[a]*

5

6 *[a]Department of Veterinary Medicine, Università Degli Studi di Milano, Via*
7 *dell'Università 6, 26900, Lodi, Italy*

8

9 [*]Corresponding author: Gabriela Ávila Morales

10 E-mail address: gabriela.avila@unimi.it

11 Department of Veterinary Medicine, Università Degli Studi di Milano, Via
12 dell'Università 6, 26900, Lodi, Italy

13 Tel: 0039 02 50334531

14

15

16 **Highlights**

17

18 - CP did not affect chicken monocytes' apoptosis and viability
19 - CP inhibited the chemotactic activity of chicken monocytes
20 - CP reduced the phagocytic activity of chicken monocytes
21 - CP exerts *in vitro* immunomodulatory effects in poultry

22

23

24

25

ABSTRACT

Pectin is a dietary fibre composed of galacturonic acid, primarily found in the citrus fruits' cell walls. Citrus pectin (CP) has demonstrated antioxidative, anticancer, and anti-inflammatory properties in humans and animals. In broilers, CP supplementation improves energy utilization and nutrient digestibility, but limited information on its effects on chicken immunity is available so far. This study aimed to assess the *in vitro* impact of CP on chicken monocytes' immune response. Cells were purified from whole blood of healthy chickens and incubated with increasing concentrations (0, 0.25, 0.5, 0.75, 1 mg/mL) of CP to determine CP working concentration. The effects of different CP concentrations on cells' apoptosis and viability were assessed by measuring caspase-3 and -7 and the cells' metabolic activity (MTT assay), respectively. CP had no dose-dependent effect on monocyte apoptosis and viability.Then, the effects of CP (0.5 mg/mL) on chicken monocytes' chemotaxis and phagocytosis were assessed by measuring transwell migration and fluorescein-labelled *E. coli* incorporation, respectively. CP inhibited both monocytes' chemotaxis and phagocytosis.These data demonstrate that CP exerts an immunomodulatory role in chicken monocytes, supporting its integration in nutrition strategies that might be beneficial for the animal's immunity and health.

Keywords: chicken, citrus pectin, monocyte, anti-inflammatory, apoptosis, chemotaxis

Abbreviations

50 CP, citrus pectin; MTT, 3-(4,5- dimethyl thiazol -2-yl)-2,5-diphenyl tetrazolium bromide;
51 DM, methyl esterification; TLR, toll-like receptor; HBSS, hank's balanced salt solution;
52 ZAS, zymosan activated serum; MCP, modified citrus pectin.
53
54 **1. Introduction**
55
56 Pectin is a family of complex polysaccharides, primarily composed of repeating units of
57 galacturonic acid, joined by $\alpha 1 \rightarrow 4$ glycosidic linkages, creating a linear polymer (Ridley
58 et al., 2001). Different degrees of methyl esterification (DM) of the pectin carboxyl
59 groups can be observed, often associated with structural and functional differences of the
60 pectin (Chen et al., 2006; Salman et al., 2008).
61 Pectin is found in all plants' cell walls, but it is most abundant in citrus fruits
62 (Sahasrabudhe et al., 2018). Citrus pectin (CP) is widely used as dietary fibre in both
63 human and animal nutrition (Langhout and Schutte, 1996; Leclere et al., 2013). Previous
64 *in vitro* and *in vivo* studies demonstrated CP's antioxidative (Sanders et al., 2004), anti-
65 diabetic (Liu et al., 2016), anticancer (Glinsky and Raz, 2009; Salehi et al., 2018), anti-
66 inflammatory (Sahasrabudhe et al., 2018; Salman et al., 2008) and other
67 immunomodulatory activities (Chen et al., 2006). *In vitro* studies performed in human
68 PBMC demonstrated that CP with higher esterification degrees (60 and 90%) inhibits in
69 a dose-dependent manner the production of the proinflammatory cytokine IL-1β, while
70 increases the secretion of anti-inflammatory cytokines IL-1ra and IL-10 (Salman et al.,
71 2008). Low DM citrus pectin blocked immune receptors in human dendritic cells and
72 murine macrophages by inhibiting the Toll-like receptor 1 (TLR1) and Toll-like receptor
73 2 (TLR2) proinflammatory pathways. The production of IL-6 and IL-10 was also reduced
74 in human dendritic cells, and only of IL-6 in murine macrophages (Sahasrabudhe et al.,

75 2018). Lemon pectin can also activate human THP-1 monocytic cell line, in a TLR and
76 DM dependent manner, and exert a protective effect on the human epithelial barrier (Vogt
77 et al., 2016). The immunomodulatory effects observed in these studies were all dependent
78 on the DM of the citrus pectin used, in which generally the higher the DM (> 50%), the
79 stronger the effects observed.
80 Although CP supplementation of broilers' diet improves the energy utilization, nutrient
81 digestibility, increasing productive performance (Silva et al., 2013), limited information
82 of CP effects on chicken immunity is available so far. The impact of CP on immune
83 functions of chicken mononuclear cells have not been assessed yet either. The present
84 investigation aims to cover this gap by evaluating the *in vitro* impact of CP (55-70% DM)
85 on chicken monocytes' immune response, including its effects on spontaneous apoptosis,
86 viability, chemotaxis and phagocytosis.
87
88 ## 2. Materials and methods
89
90 ### *2.1. Materials*
91
92 #### *2.1.1. Chemicals*
93 Methylcellulose, EDTA, NaCl (Sigma, St. Louis, USA) were used for PBMC isolation.
94 Pectin esterified (55-70%) potassium salt from citrus fruit, cell tested, (Sigma, St. Louis,
95 USA) was used for treating the cells.
96
97 #### *2.1.2. Reagents*
98 Ficoll-Paque PLUS (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden), Percoll, red
99 blood cell lysis buffer, sterile-filtered PBS and HBSS, endotoxin-free water (Sigma, St.

100 Louis, USA) were used for PBMC isolation. Cells were resuspended in complete
101 medium, comprising RPMI 1640 Medium with 25 Mm HEPES and L-Glutamine (Sigma,
102 St. Louis, USA), supplemented with 1% of Non-essential Amino Acid Solution 100X and
103 1% Penicillin Streptomycin Solution 100X (Euroclone, Milano, Italy), and 10% FBS
104 (Sigma, St. Louis, USA).
105 Apo-ONE® Homogeneous Caspase-3/7 Assay (Promega, Milano, Italy) and Cell
106 Proliferation Kit I (MTT) (Roche, Mannheim, Germany) were used for apoptosis and
107 viability assays, respectively. Chemotaxis and phagocytosis were measured by using
108 Zymosan A from *Saccharomyces cerevisiae* and fluorescein-labelled *Escherichia coli*
109 bioparticles K-12 strain (Invitrogen, Oregon, USA), respectively.
110
111 *2.2. Purification of monocytes from blood*
112
113 Chicken monocytes were isolated from peripheral blood through double discontinuous
114 gradient centrifugation, as previously described for chicken (He et al., 2007; Kogut et al.,
115 1995), with few modifications. Briefly, fifty mL of peripheral blood from 42 days-old
116 hybrid broilers (ROSS 308) were collected during routine slaughtering procedures at a
117 local slaughterhouse and pooled in sterile flasks containing 0.2% EDTA per mL of blood
118 as anticoagulant. The blood was mixed (1.5:1 v/v) with methylcellulose 1% (25
119 centiposes) and centrifuged at 40g without brakes for 30 min at 4°C. The supernatant was
120 then collected, diluted (1:1) with PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA and layered
121 over a double discontinuous Ficoll-Percoll gradient (specific gravity 1.077 g/mL of Ficoll
122 over 1.119 g/mL of Percoll) in 15 mL conical centrifuge tubes, and centrifuged at 200g
123 without brakes for 30 min at 4°C. The PBMC ring was collected at the Ficoll/supernatant
124 interface, washed twice with PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA and centrifuged

125 at 200g for 10 min at 4°C to remove the contaminating thrombocytes. For red blood cells
126 elimination, Red Blood Cell Lysis Buffer was added to the cells for 3 min at room
127 temperature, and cells were washed with PBS without Ca^{2+} and Mg^{2+} + 2mM EDTA until
128 the supernatant was clear. Finally, the PBMC were counted, and their viability assessed
129 with trypan blue exclusion. The purification of monocytes was carried out by allowing
130 the PBMC to adhere for 2 h at room temperature to the cell culture treated plates of each
131 assay, as previously described (He et al., 2007). Non-adherent cells were removed, and
132 monocytes were washed with complete warm medium. The monocytes' purity was
133 determined by microscopic examination of Diff-Quick stained cytospin smears. Cell
134 viability, measured with the Trypan Blue exclusion method, was higher than 95%. At the
135 microscope examination, monocyte purity was of 70% approximately.

136

137 *2.3.Citrus pectin preparation*

138

139 Citrus pectin esterified (55-70%) potassium salt was reconstituted in endotoxin-free water
140 at a concentration of 10 mg/mL. Briefly, 2 mL of endotoxin-free water were added to 20
141 mg of CP, and the solution was then vortexed thoroughly until it was dissolved. The CP
142 solution was filtered with 0.22 μm filters. Aliquots from the stock solution were prepared
143 and stored at 4°C until use. Working dilutions with complete medium were freshly
144 prepared. The amounts of CP used for determining its working concentration were 0,
145 0.25, 0.5, 0.75, 1 mg/mL. Once selected the CP working solution, a concentration of 0.5
146 mg/mL was used to measure chemotaxis and phagocytosis.

147

148 *2.4.Viability assay (MTT)*

149

150 To assess the potential toxicity of CP on chicken monocytes, the cells' viability was
151 evaluated by using the Cell Proliferation Kit I (MTT), as previously reported (Catozzi et
152 al., 2020). The experiment was carried out on cells purified from 3 different pools,
153 consisting of 9 animals each. A total of 1×10^5 PBMC (100 µl) were seeded in duplicate
154 in 96-well sterile plates MICROTEST (Becton Dickinson and Company, Franklin Lake,
155 USA) to let the monocytes adhere to the plates. After removing the non-adherent cells,
156 monocytes were incubated 20 h with increasing CP concentrations (0.25, 0.5, 0.75 and 1
157 mg/mL) or with medium (control) at 41°C in humified atmosphere 5% CO_2. After the
158 incubation period, 10 µl of MTT labelling reagent was added to each well and incubated
159 at 41°C for 4 h. The formazan crystals were solubilized by adding 100 µl of the
160 solubilization buffer and incubating at 41°C overnight, following the manufacturer's
161 instructions. Finally, the absorbance was read at 550 nm with LabSystems Multiskan
162 plate reader Spectrophotometer.
163
164 *2.5.Apoptosis assay*
165
166 Apoptosis assay was performed in duplicates; 5×10^4 PBMC were seeded in 384-well
167 black plates (Costar, Corning, USA). The experiment was carried out on the adhered
168 monocytes purified from 4 different pools of 9 animals each. To determine CP working
169 concentration, the cells were incubated for 20 h at 41°C in a humified atmosphere of 5%
170 CO_2 with increasing CP concentrations (0.25, 0.5, 0.75 and 1 mg/mL) medium as control
171 and the spontaneous apoptosis was measured. The apoptosis was measured after 20 h of
172 incubation by using the Apo-ONE® Homogeneous Caspase-3/7 kit. The caspase-3/7
173 reagent was added to each well, and the fluorescence intensity was measured using a
174 fluorescence plate reader Fluoroscan Ascent at 485/538 nm (absorbance/emission), every

175 30 minutes up to 2 hours and after overnight incubation, as previously described in bovine
176 monocytes with minor modifications (Ceciliani et al., 2007).
177
178 *2.6.Chemotaxis assay*
179
180 Monocytes chemotaxis towards zymosan activated serum (ZAS) was measured as
181 previously reported (Ávila et al., 2020; Lecchi et al., 2008), with minor changes. The
182 experiment was performed on cells purified from 5 different pools, consisting of 9
183 animals each. Monocytes were first pretreated overnight, in the absence of
184 chemoattractant, with 0.5 mg/mL of CP or migration medium only (RMPI-1640 with 1%
185 of FBS) as a control (vehicle) in 24-well Transwell migration plates (Costar, Corning,
186 USA), equipped with an 8 μm pore size membrane. First, a total number of $1x10^5$ PBMC
187 (100 μl) were added in duplicates in the upper chamber, and monocytes were allowed to
188 adhere for 2 h at room temperature to the upper insert membrane of the transwell plate.
189 After incubation, non-adherent cells were removed and monocytes washed with the warm
190 medium. Adhered monocytes were pretreated with 0.5 mg/ mL of CP or with migration
191 medium by adding 100 μl of CP or medium to the upper chamber and 650 μl to the lower
192 chamber, for 20 h at 41°C in a humified atmosphere of 5% CO_2. After pretreating the
193 cells, chemotaxis was measured by adding 3 mg/mL of the chemoattractant ZAS to the
194 lower chamber, in the presence of newly added CP (0.5 mg/mL) or migration medium
195 (vehicle) in both chambers, and again incubated for 2 h at 41°C in a humified atmosphere
196 of 5% CO_2. Cells incubated with migration medium but without ZAS were considered as
197 the negative control. Upper chambers were removed, and non-migrated cells on the upper
198 part of the membrane were eliminated using a cotton swab moistured with PBS. Finally,
199 migrated cells were stained with Diff-Quick (Sigma, St Louis, USA) and counted in ten

200 different fields, using light microscopy (inverted microscope). The chemoattractant ZAS
201 was prepared as previously described in bovine monocytes (Lecchi et al., 2008), using
202 chicken serum.
203
204 *2.7.Phagocytosis assay*
205
206 Monocytes' phagocytosis was determined as previously described (Lecchi et al., 2011).
207 The experiment was carried out on cells purified from 6 different pools, consisting of 9
208 animals each. In a first step, $6x10^8$ fluorescein-labelled *Escherichia coli* bioparticles (K-
209 12 strain) were opsonized with 20% of chicken serum for 30 min at 37°C. Opsonized
210 bacteria were centrifuged at 800 g for 15 min, suspended in HBSS and stored at -20°C
211 until use. A total of $3x10^5$ PBMC (100 µl) were seeded in duplicates in 96-well sterile
212 plates for monocyte isolation for 2 h at room temperature. After removing non-adherent
213 cells and washing the monocytes with warm HBSS, 100 µl of 0.5 mg/mL CP or only
214 medium (vehicle) as the control was added to each well. The medium was added to reach
215 a final volume of 200 µl, and cells were incubated for 20 h at 41°C in a humidified
216 atmosphere of 5% CO_2. Cells were then washed with sterile HBSS, and a total of 45
217 opsonized fluorescein-labelled *E. coli* bioparticles (100 µl) per well were added.
218 Monocytes were incubated again for 2 h at 41°C in a humidified atmosphere of 5% CO_2.
219 Cells were washed twice with HBSS and 0.4% of trypan blue was added to quench the
220 fluorescence from non-internalized bacteria. Cells were finally washed with HBSS and
221 the fluorescence intensity of fluorescein-labelled *E. coli* bioparticles was measured using
222 a Fluoroscan Ascent at 485/538 nm (absorbance/emission).
223
224 *2.8.Statistical analyses*

225

226 Statistical analyses were performed in GraphPad Prism 8.0.2. Data normality of
227 apoptosis, viability and chemotaxis was assessed by applying the Shapiro Wilk test and
228 the Kolmogorov Smirnov test for phagocytosis. For the assessment of CP's effect on the
229 apoptosis, viability and chemotaxis, repeated measures one-way ANOVA and Tukey’s
230 multiple comparison tests were used for normally distributed samples and a one-tailed
231 paired t-test for phagocytosis. Statistical differences were accepted at $P \leq 0.05$.

232

233 **3. Results and discussion**

234

235 In the present study, we reported the effects of CP on chicken monocytes’ immune
236 functions, including chemotaxis and phagocytosis. To test the impact of CP on
237 monocytes, viability and apoptosis were also measured.
238 The purity of the isolated monocytes was approximately 70%, the contaminant cells being
239 mostly thrombocytes, which is consistent with what was reported in previous studies (Ma
240 et al., 2019; Reddy et al., 2016). Since avian thrombocytes are nucleated cells with sizes
241 and densities similar to lymphocytes and monocytes, they are often purified together with
242 them. Moreover, their capacity to adhere to the plastic makes their complete depletion
243 from monocytes cultures challenging (Mudroňová et al., 2014).
244 Pectin has shown immunomodulatory roles by preventing immunological diseases like
245 asthma (Zhang et al., 2016), allergies, colitis and inflammatory bowel diseases through
246 directly regulating inflammation in several animal models (Ishisono et al., 2019;
247 Sahasrabudhe et al., 2018). In broilers, including CP in the diet has improved the animals’
248 growth’s performance (Silva et al., 2013), but information about its immunomodulatory
249 activity in chickens is still absent.

250 As the first step of this study, we measured chicken monocytes' viability and apoptosis
251 to determine the optimal working concentration of CP and to evaluate potential CP
252 cytotoxic effects. Cells' viability (Fig. 1A) and spontaneous apoptosis (Fig. 1B) were not
253 affected by increasing CP concentrations. Apoptosis plays a critical role in regulating and
254 shaping the immune system, as it maintains cellular homeostasis by eliminating the
255 excess of immune cells (Feig and Peter, 2007). Therefore, apoptosis can be regarded as a
256 way of controlling immune cells' activity in the inflammatory focus. Modified citrus
257 pectin (MCP) can induce apoptosis of breast (Salehi et al., 2018), prostate (Jackson et al.,
258 2007) and colon (Olano-Martin et al., 2003) cancer cells, without damaging normal cells
259 even at high concentrations (600 μg/mL) (Chauhan et al., 2005). The same increasing
260 concentrations of CP (0.25, 0.5, 0.75 and 1 mg/mL) herein tested, had also no cytotoxic
261 effects on a macrophage murine cell line (RAW264.7) (Chen et al., 2006), suggesting that
262 the impact of CP might be cell-specific.
263 In the second step of this investigation, CP's effect (0.5 mg/mL) on the chemotaxis of
264 chicken monocytes was assessed. This concentration was used throughout the next
265 experiments, as monocytes' viability and apoptosis remained unchanged at this
266 concentration, and no other cytotoxic effects in immune cells, including macrophages,
267 have been described (Fan et al., 2018). On the contrary, several immunomodulatory
268 effects, specifically anti-inflammatory ones, have been observed (Chen et al., 2006;
269 Salman et al., 2008).
270 The activation of medium treated (vehicle) cells with ZAS enhanced the chemotaxis of
271 monocytes compared to the negative control (cells without ZAS) ($P < 0.01$). Co-
272 incubation with CP decreased ($P = 0.03$) chemotaxis rate of cells compared to ZAS
273 activated cells treated with medium (vehicle), while no differences were observed
274 compared to cells without ZAS (negative control) (Fig. 2). The present findings

275 demonstrated that CP reduces the *in vitro* monocytes' chemotaxis in chicken. These
276 results are consistent with those reported in cancer cells, which revealed that smaller sizes
277 of MCP fractions inhibit their migration (do Prado et al., 2019). A similar inhibitory effect
278 on the migration of a murine fibroblasts treated with 0.5 mg/mL of ginseng pectin was
279 observed by changing the cells' morphology and organization of actin filaments, and by
280 reducing cell adhesion and spreading to the substratum (Fan et al., 2018). Specifically,
281 MCP inhibited cancer and endothelial cells' migration through the direct inhibition of
282 Galectin-3, a carbohydrate-binding protein expressed by numerous cell types, including
283 monocytes, which is involved in cell migration and phagocytosis (Nangia-Makker et al.,
284 2002; Simon and Green, 2005). Indeed, pectins can modulate directly innate immune
285 responses by interacting with monocytes and macrophages' pattern recognition receptors
286 (PPR) like Galectin-3 and Toll-like receptors (TLR) (Beukema et al., 2020). The direct
287 inhibition of Galectin-3 and TLR2, TLR1 and TLR4 by CP (Ishisono et al., 2017;
288 Sahasrabudhe et al., 2018) could explain the suppression of some monocytes' immune
289 responses and consequently of its anti-inflammatory effects. On the other hand, after their
290 binding, pectins can also present TLR2/4 activating properties, and as reported
291 previously, TLR2/4 signalling can also cause a rapid arrest of human monocytes'
292 chemotaxis (Yi et al., 2012).
293 The last set of experiments was performed to explore CP's capacity in modulating chicken
294 monocytes' phagocytosis. Monocytes were treated with CP (0.5 mg/mL) or complete
295 medium (vehicle) as control for 20 h at 41°C, and their phagocytic capability was
296 measured. Fig. 3 shows a reduction ($P = 0.03$) in the phagocytosis of CP treated
297 monocytes when compared to the control. These results evidence that CP inhibited
298 another important monocyte defensive function, phagocytosis. This result differs from
299 those previously reported, showing that a high DM pectin (around 85% DM) isolated

300 from berries increased peritoneal macrophages' phagocytosis (Wang et al., 2015).
301 However, contrasting results of pectin's immunomodulatory effects on leukocytes are
302 often reported, as the content of galacturonic acid and the degree of DM determine
303 pectin's ability to decrease immune reactivity. Pectins with higher than 80% of
304 galacturonic acid decreased the *in vivo* accumulation of murine macrophages, while
305 increasing or no effects were seen with pectins with less than 75% of galacturonic acid
306 (Popov and Ovodov, 2013). On the contrary, CP with lower DM showed anti-
307 inflammatory properties in mice *in vivo*, a feature lost as their DM increases (Popov et
308 al., 2013).

309 Finally, the suppression of chicken monocytes' phagocytosis could also be explained by
310 the inhibition of Galectin-3, which plays a significant role in macrophage phagocytosis,
311 as a major component of phagosomes and phagocytic cups (Sano et al., 2003).

312 In conclusion, this is the first study to indicate that CP inhibits two main inflammatory
313 functions of chicken monocytes', namely chemotaxis and phagocytosis, suggesting
314 potential anti-inflammatory roles. Moreover, our results evidence that the effects of CP
315 on apoptosis and viability are cell-specific, as the pro-apoptotic effects often observed in
316 cancer cells were not seen on chicken monocytes. Altogether, these results reinforce the
317 concept that CP may play an essential role in immunity, specifically in dampening
318 inflammation. Such potential anti-inflammatory functions make CP an attractive dietary
319 fibre for novel nutrition strategies to enhance the animals' health. Besides, these results
320 highlight the importance to formulate balanced diets in chicken, specially considering
321 that an excessive CP content may induce an immunosuppressive status, increasing the
322 risk of developing opportunistic diseases. Further analyses on other inflammatory
323 immune functions (e.g., oxidative burst, killing capability and cytokine production) and

324 integration of system biology approaches might be highly valuable to elucidate CP
325 biological significance.
326
327 **Conflict of interest statement**
328
329 None of the authors of this paper has a financial or personal relationship with other people
330 or organizations that could inappropriately influence or bias the paper's content.
331
332 **Acknowledgements**
333
334 We acknowledge the support of Valentina Lodde and her team of the Dipartimento di
335 Scienze Veterinarie per la Salute, la Produzione Animale e la Sicurezza Alimentare at the
336 Università degli Studi di Milano,–Italy, for her valuable support in the microscopic
337 evaluation and digital imaging acquisition of chicken thrombocytes and monocytes; and
338 to Rodrigo Guabiraba-Brito, from the Infection and Innate Immunity in Monogastric
339 Livestock Department, Centre INRAE, Val de Loire, Nouzilly, France, for his valuable
340 support in chicken PBMC isolation and thrombocyte elimination.
341
342 **Funding**
343
344 This study was supported by the European Union's Horizon 2020 research and innovation
345 programme H2020-MSCA- ITN-2017- EJD: Marie Skłodowska-Curie Innovative
346 Training Networks (European Joint Doctorate) [Grant agreement nº: 765423, 2017] –
347 MANNA.
348

349 **References**

350

351 Ávila, G., Catozzi, C., Pravettoni, D., Sala, G., Martino, P., Meroni, G., Lecchi, C.,
352 Ceciliani, F., 2020. In vitro effects of conjugated linoleic acid (CLA) on
353 inflammatory functions of bovine monocytes. J. Dairy Sci. 103, 8554–8563.
354 https://doi.org/10.3168/jds.2020-18659

355 Beukema, M., Faas, M.M., de Vos, P., 2020. The effects of different dietary fiber pectin
356 structures on the gastrointestinal immune barrier: impact via gut microbiota and
357 direct effects on immune cells. Exp. Mol. Med. 52, 1364–1376.
358 https://doi.org/10.1038/s12276-020-0449-2

359 Catozzi, C., Ávila, G., Zamarian, V., Pravettoni, D., Sala, G., Ceciliani, F., Lacetera, N.,
360 Lecchi, C., 2020. In-vitro effect of heat stress on bovine monocytes lifespan and
361 polarization. Immunobiology 225, 151888.
362 https://doi.org/https://doi.org/10.1016/j.imbio.2019.11.023

363 Ceciliani, F., Pocacqua, V., Miranda-Ribera, A., Bronzo, V., Lecchi, C., Sartorelli, P.,
364 2007. α1-Acid glycoprotein modulates apoptosis in bovine monocytes. Vet.
365 Immunol. Immunopathol. 116, 145–152.
366 https://doi.org/10.1016/j.vetimm.2007.01.006

367 Chauhan, D., Li, G., Podar, K., Hideshima, T., Neri, P., He, D., Mitsiades, N., Richardson,
368 P., Chang, Y., Schindler, J., Carver, B., Anderson, K.C., 2005. A Novel
369 Carbohydrate-Based Therapeutic GCS-100 Overcomes Bortezomib Resistance and
370 Enhances Dexamethasone-Induced Apoptosis in Multiple Myeloma Cells. Cancer
371 Res. 65, 8350 LP – 8358. https://doi.org/10.1158/0008-5472.CAN-05-0163

372 Chen, C.H., Sheu, M.T., Chen, T.F., Wang, Y.C., Hou, W.C., Liu, D.Z., Chung, T.C.,
373 Liang, Y.C., 2006. Suppression of endotoxin-induced proinflammatory responses by

374 citrus pectin through blocking LPS signaling pathways. Biochem. Pharmacol. 72,
375 1001–1009. https://doi.org/10.1016/j.bcp.2006.07.001
376 do Prado, S.B.R., Shiga, T.M., Harazono, Y., Hogan, V.A., Raz, A., Carpita, N.C., Fabi,
377 J.P., 2019. Migration and proliferation of cancer cells in culture are differentially
378 affected by molecular size of modified citrus pectin. Carbohydr. Polym. 211, 141–
379 151. https://doi.org/https://doi.org/10.1016/j.carbpol.2019.02.010
380 Fan, Y., Sun, L., Yang, S., He, C., Tai, G., Zhou, Y., 2018. The roles and mechanisms of
381 homogalacturonan and rhamnogalacturonan I pectins on the inhibition of cell
382 migration. Int. J. Biol. Macromol. 106, 207–217.
383 https://doi.org/https://doi.org/10.1016/j.ijbiomac.2017.08.004
384 Feig, C., Peter, M.E., 2007. How apoptosis got the immune system in shape. Eur. J.
385 Immunol. 37 Suppl 1, S61-70. https://doi.org/10.1002/eji.200737462
386 Glinsky, V. V., Raz, A., 2009. Modified citrus pectin anti-metastatic properties: one
387 bullet, multiple targets. Carbohydr. Res. 344, 1788–1791.
388 https://doi.org/10.1016/j.carres.2008.08.038
389 He, H., Genovese, K.J., Nisbet, D.J., Kogut, M.H., 2007. Synergy of CpG
390 oligodeoxynucleotide and double-stranded RNA (poly I:C) on nitric oxide induction
391 in chicken peripheral blood monocytes. Mol. Immunol. 44, 3234–3242.
392 https://doi.org/10.1016/j.molimm.2007.01.034
393 Ishisono, K., Mano, T., Yabe, T., Kitaguchi, K., 2019. Dietary Fiber Pectin Ameliorates
394 Experimental Colitis in a Neutral Sugar Side Chain-Dependent Manner. Front.
395 Immunol. 10, 2979. https://doi.org/10.3389/fimmu.2019.02979
396 Ishisono, K., Yabe, T., Kitaguchi, K., 2017. Citrus pectin attenuates endotoxin shock via
397 suppression of Toll-like receptor signaling in Peyer's patch myeloid cells. J. Nutr.
398 Biochem. 50, 38–45.

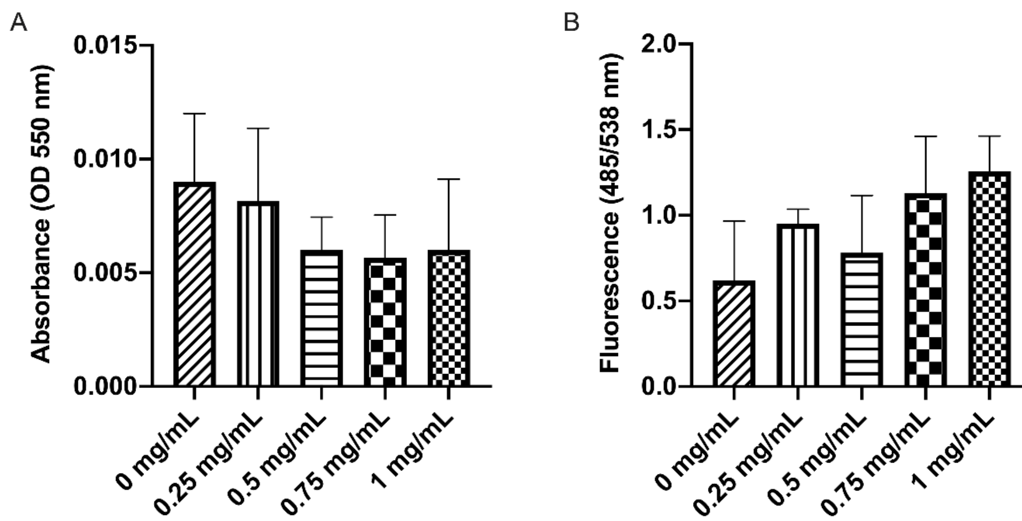
399 Jackson, C.L., Dreaden, T.M., Theobald, L.K., Tran, N.M., Beal, T.L., Eid, M., Gao,
400 M.Y., Shirley, R.B., Stoffel, M.T., Kumar, M.V., Mohnen, D., 2007. Pectin induces
401 apoptosis in human prostate cancer cells: correlation of apoptotic function with
402 pectin structure. Glycobiology 17, 805–819.
403 https://doi.org/10.1093/glycob/cwm054
404 Kogut, M.H., McGruder, E.D., Hargis, B.M., Corner, D.E., DeLoach, J.R., 1995. In vivo
405 activation of heterophil function in chickens following injection with Salmonella
406 enteritidis-immune lymphokines. J. Leukoc. Biol. 57, 56–62.
407 Langhout, D.J., Schutte, J.B., 1996. Nutritional Implications of Pectins in Chicks in
408 Relation to Esterification and Origin of Pectins. Poult. Sci. 75, 1236–1242.
409 https://doi.org/10.3382/ps.0751236
410 Lecchi, C., Ceciliani, F., Bernasconi, S., Franciosi, F., Bronzo, V., Sartorelli, P., 2008.
411 Bovine alpha-1 acid glycoprotein can reduce the chemotaxis of bovine monocytes
412 and modulate CD18 expression. Vet. Res. 39.
413 https://doi.org/10.1051/vetres:2008027
414 Lecchi, C., Invernizzi, G., Agazzi, A., Ferroni, M., Pisani, L.F., Savoini, G., Ceciliani, F.,
415 2011. In vitro modulation of caprine monocyte immune functions by ω-3
416 polyunsaturated fatty acids. Vet. J. 189, 353–355.
417 https://doi.org/10.1016/j.tvjl.2010.09.001
418 Leclere, L., Cutsem, P. Van, Michiels, C., 2013. Anti-cancer activities of pH- or heat-
419 modified pectin. Front. Pharmacol. 4 OCT, 1–8.
420 https://doi.org/10.3389/fphar.2013.00128
421 Liu, Y., Dong, M., Yang, Z., Pan, S., 2016. Anti-diabetic effect of citrus pectin in diabetic
422 rats and potential mechanism via PI3K/Akt signaling pathway. Int. J. Biol.
423 Macromol. 89, 484–488.

424 https://doi.org/https://doi.org/10.1016/j.ijbiomac.2016.05.015
425 Ma, Y., Liang, Y., Wang, N., Cui, L., Chen, Z., Wu, H., Zhu, C., Wang, Z., Liu, S., Li,
426 H., 2019. Avian Flavivirus Infection of Monocytes/Macrophages by Extensive
427 Subversion of Host Antiviral Innate Immune Responses. J. Virol. 93, e00978-19.
428 https://doi.org/10.1128/JVI.00978-19
429 Mudroňová, D., Kožár, M., Molnár, L., Trbolová, A., 2014. Viability and discrimination
430 of avian peripheral blood mononuclear cells and thrombocytes intended for
431 improvement of wound healing in birds. Acta Vet. Hung. 62, 334–339.
432 https://doi.org/10.1556/AVet.2014.014
433 Nangia-Makker, P., Hogan, V., Honjo, Y., Baccarini, S., Tait, L., Bresalier, R., Raz, A.,
434 2002. Inhibition of Human Cancer Cell Growth and Metastasis in Nude Mice by
435 Oral Intake of Modified Citrus Pectin. JNCI J. Natl. Cancer Inst. 94, 1854–1862.
436 https://doi.org/10.1093/jnci/94.24.1854
437 Olano-Martin, E., Rimbach, G.H., Gibson, G.R., Rastall, R.A., 2003. Pectin and pectic-
438 oligosaccharides induce apoptosis in in vitro human colonic adenocarcinoma cells.
439 Anticancer Res. 23, 341–346.
440 Popov, S. V., Ovodov, Y.S., 2013. Polypotency of the immunomodulatory effect of
441 pectins. Biochem. 78, 823–835. https://doi.org/10.1134/S0006297913070134
442 Popov, S. V, Markov, P.A., Popova, G.Y., Nikitina, I.R., Efimova, L., Ovodov, Y.S.,
443 2013. Anti-inflammatory activity of low and high methoxylated citrus pectins.
444 Biomed. Prev. Nutr. 3, 59–63.
445 https://doi.org/https://doi.org/10.1016/j.bionut.2012.10.008
446 Reddy, V.R.A.P., Trus, I., Desmarets, L.M.B., Li, Y., Theuns, S., Nauwynck, H.J., 2016.
447 Productive replication of nephropathogenic infectious bronchitis virus in peripheral
448 blood monocytic cells, a strategy for viral dissemination and kidney infection in

449 chickens. Vet. Res. 47, 70. https://doi.org/10.1186/s13567-016-0354-9
450 Ridley, B.L., O'Neill, M.A., Mohnen, D., 2001. Pectins: structure, biosynthesis, and
451 oligogalacturonide-related signaling. Phytochemistry 57, 929–967.
452 https://doi.org/https://doi.org/10.1016/S0031-9422(01)00113-3
453 Sahasrabudhe, N.M., Beukema, M., Tian, L., Troost, B., Scholte, J., Bruininx, E.,
454 Bruggeman, G., van den Berg, M., Scheurink, A., Schols, H.A., Faas, M.M., de Vos,
455 P., 2018. Dietary fiber pectin directly blocks toll-like receptor 2-1 and prevents
456 doxorubicin-induced ileitis. Front. Immunol. 9, 1–19.
457 https://doi.org/10.3389/fimmu.2018.00383
458 Salehi, F., Behboudi, H., Kavoosi, G., Ardestani, S.K., 2018. Oxidative DNA damage
459 induced by ROS-modulating agents with the ability to target DNA: A comparison
460 of the biological characteristics of citrus pectin and apple pectin. Sci. Rep. 8, 13902.
461 https://doi.org/10.1038/s41598-018-32308-2
462 Salman, H., Bergman, M., Djaldetti, M., Orlin, J., Bessler, H., 2008. Citrus pectin affects
463 cytokine production by human peripheral blood mononuclear cells. Biomed.
464 Pharmacother. 62, 579–582. https://doi.org/10.1016/j.biopha.2008.07.058
465 Sanders, L.M., Henderson, C.E., Hong, M.Y., Barhoumi, R., Burghardt, R.C., Wang, N.,
466 Spinka, C.M., Carroll, R.J., Turner, N.D., Chapkin, R.S., Lupton, J.R., 2004. An
467 Increase in Reactive Oxygen Species by Dietary Fish Oil Coupled with the
468 Attenuation of Antioxidant Defenses by Dietary Pectin Enhances Rat Colonocyte
469 Apoptosis. J. Nutr. 134, 3233–3238. https://doi.org/10.1093/jn/134.12.3233
470 Sano, H., Hsu, D.K., Apgar, J.R., Yu, L., Sharma, B.B., Kuwabara, I., Izui, S., Liu, F.-T.,
471 2003. Critical role of galectin-3 in phagocytosis by macrophages. J. Clin. Invest.
472 112, 389–397. https://doi.org/10.1172/JCI17592
473 Silva, V.K., de Souza Morita, V., Boleli, I.C., 2013. Effect of pectin extracted from citrus

474 pulp on digesta characteristics and nutrient digestibility in broilers chickens. Rev.
475 Bras. Zootec. 42, 575–583. https://doi.org/10.1590/S1516-35982013000800007
476 Simon, S.I., Green, C.E., 2005. Molecular Mechanics and Dynamics of Leukocyte
477 Recruitment During Inflammation. Annu. Rev. Biomed. Eng. 7, 151–185.
478 https://doi.org/10.1146/annurev.bioeng.7.060804.100423
479 Vogt, L.M., Sahasrabudhe, N.M., Ramasamy, U., Meyer, D., Pullens, G., Faas, M.M.,
480 Venema, K., Schols, H.A., de Vos, P., 2016. The impact of lemon pectin
481 characteristics on TLR activation and T84 intestinal epithelial cell barrier function.
482 J. Funct. Foods 22, 398–407.
483 https://doi.org/https://doi.org/10.1016/j.jff.2016.02.002
484 Wang, H., Gao, T., Du, Y., Yang, H., Wei, L., Bi, H., Ni, W., 2015. Anticancer and
485 immunostimulating activities of a novel homogalacturonan from Hippophae
486 rhamnoides L. berry. Carbohydr. Polym. 131, 288–296.
487 https://doi.org/https://doi.org/10.1016/j.carbpol.2015.06.021
488 Yi, L., Chandrasekaran, P., Venkatesan, S., 2012. TLR Signaling Paralyzes Monocyte
489 Chemotaxis through Synergized Effects of p38 MAPK and Global Rap-1 Activation.
490 PLoS One 7, e30404.
491 Zhang, Z., Shi, L., Pang, W., Liu, W., Li, J., Wang, H., Shi, G., 2016. Dietary Fiber Intake
492 Regulates Intestinal Microflora and Inhibits Ovalbumin-Induced Allergic Airway
493 Inflammation in a Mouse Model. PLoS One 11, e0147778.
494

495 **Figures**



496

497 **Fig. 1.** *In vitro* effect of increasing concentrations (0.25, 0.5, 0.75 and 1 mg/mL) of citrus
498 pectin (CP) on chicken monocytes' (A) viability and (B) apoptosis. 3-(4,5- dimethyl
499 thiazol -2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction by metabolic active
500 cells and caspase-3/7 enzymatic activity of chicken monocytes treated with citrus pectin
501 or medium as control (0 mg/mL) were measured for viability and apoptosis, respectively.
502 Data are means ± SEM of three and four animals, for viability and apoptosis, respectively.
503 All experiments were carried out using duplicates for each condition.

504

505

506

507

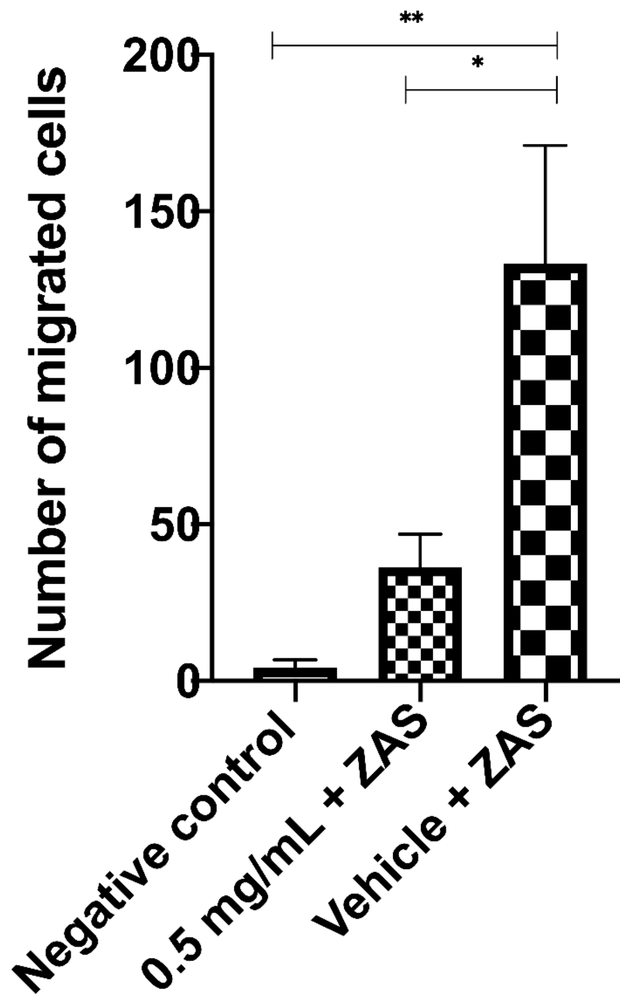
508

509

510

511

512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527



528 **Fig. 2.** Effects of citrus pectin (CP) on chicken monocytes chemotaxis. Citrus pectin (0.5
529 mg/mL) and vehicle control (only medium) treated cells were activated with Zymosan
530 Activated Serum (ZAS), while negative control cells were not. Data are means ± SEM of
531 five independent experiments. All experiments were carried out using duplicates for each
532 treatment. Significance was declared for $P < 0.05$ (*).

533
534
535
536

537

538

539

540

541

542

543

544

545

546

547

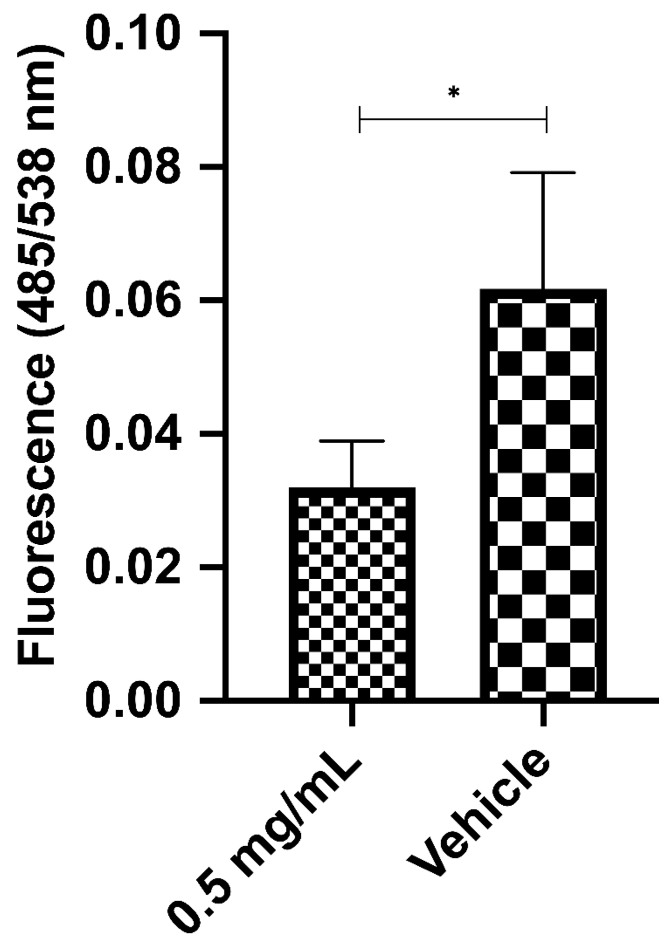
548

549

550

551

552



553 **Fig. 3.** Phagocytosis of fluorescein-labelled *Escherichia coli* bioparticles by chicken
554 monocytes after 20 h incubation with citrus pectin (0.5 mg/mL). Cells treated with only
555 medium (vehicle) were considered as control. Data are means ± SEM of six independent
556 experiments. All experiments were carried out using duplicates for each
557 treatment.Significance was declared for $P < 0.05$ (*).

558