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Immunomodulatory activity of 5 kDa permeate fractions of casein hydrolysates generated using a range of enzymes in Jurkat T cells and RAW264.7 macrophages

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ABSTRACT

| 25 | The in vitro bioactivity of 5 kDa ultrafiltration permeate fractions of casein hydrolysates |
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| 26 | produced using different enzymes were compared. Reverse phase ultra-performance liquid |
| 27 | chromatography and gel permeation chromatography showed that the permeates had different |
| 28 | physicochemical properties (molecular mass and degree of hydrolysis). The Flavourzyme® |
| 29 | permeate had the highest activity in the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic |
| 30 | acid) (ABTS) assay. Cellular antioxidant and immunomodulatory assays showed that none of |
| 31 | the permeates exhibited in vitro antioxidant activity, while all permeates significantly ($P <$ |
| 32 | 0.05) decreased interleukin-6 (IL-6) production in ConA-stimulated Jurkat T cells at 0.50% |
| 33 | (w/v) and LPS-stimulated RAW264.7 cells at 0.05 and 0.50% (w/v). Three permeates, |
| 34 | obtained using Flavourzyme [®] , Flavorpro Whey and trypsin, also significantly ($P < 0.05$) |
| 35 | decreased IL-1 β production at 0.05% (w/v) in RAW264.7 cells. Western blot analysis |
| 36 | showed that all permeates significantly decreased the expression of the NF-kB subunit, p65, |
| 37 | in RAW264.7 cells indicating that anti-inflammatory activity may be associated with this |
| 38 | pathway. |
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1. Introduction

| 43 | Cardiovascular disease (CVD), in particular atherosclerosis, is associated with |
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| 44 | elevated inflammation and oxidative stress. Drugs used to treat these conditions may produce |
| 45 | unwanted side effects, therefore natural alternatives to synthetic drugs are constantly sought |
| 46 | (Chakrabarti, Jahandideh & Wu, 2014). Bioactive peptides are small protein fragments that |
| 47 | have the potential to exert beneficial health effects in vivo (Urista, Fernández, Rodriguez, |
| 48 | Cuenca & Jurado, 2011). Bioactive peptides, derived from the milk protein casein, |
| 49 | demonstrate numerous bioactivities such as antihypertensive, opioid and antimicrobial |
| 50 | activity (Di Pierro, O'Keeffe, Poyarkov, Lomolino, & FitzGerald, 2014; Kazlauskaite et al., |
| 51 | 2005; Nongonierma, O'Keeffe, & FitzGerald, 2016; Phelan, Aherne-Bruce, O'Sullivan, |
| 52 | FitzGerald, & O'Brien, 2009; Tang et al., 2015; Trivedi, Zhang, Lopez-Toledano, Clarke, & |
| 53 | Deth, 2016; Yamada et al., 2015). The enzymes used in the generation of these peptides, as |
| 54 | well as the length and amino acid sequence of the resultant peptides influence the |
| 55 | bioactivities observed (Power, Jakeman, & FitzGerald, 2013). |
| 56 | Casein hydrolysates with cellular antioxidant activity have been reported in numerous |
| 57 | studies. Garcia-Nebot, Cilla, Alegría, and Barberá (2011) reported that |
| 58 | caseinophosphopeptides showed cyto-protective effects against H ₂ O ₂ -induced oxidative |
| 59 | stress in Caco-2 cells. Xie, Wang, Ao, and Li (2013) reported that an Alcalase® generated |
| 60 | hydrolysate protected HepG2 cells from H ₂ O ₂ -induced oxidative damage. Hydrolysis of |
| 61 | bovine case n glycomacropeptide with papain was also reported to protect against H_2O_2 - |
| 62 | induced oxidation in RAW264.7 cells, along with increasing the level of cellular antioxidant |
| 63 | enzymes (Cheng, Gao, Song, Ren, & Mao, 2015). Treatment of Jurkat T cells with casein |
| 64 | hydrolysates generated using different mammalian, plant or bacterial enzymes has previously |
| 65 | been reported to increase cellular antioxidant levels (Lahart et al., 2011; Phelan et al., 2009). |

More recently, the hydrolysis of casein using Prolyve®, generated a hydrolysate which
prevented H₂O₂-induced DNA damage in U937 cells (Cermeño, FitzGerald, & O'Brien,
2016).

69 Casein hydrolysates have also been studied for their immunomodulatory and antiinflammatory potential. The tryptic hydrolysis of casein generated a hydrolysate with 70 immune enhancing effects in mouse macrophages (Kazlauskaite et al., 2005), while β-casein 71 and several peptides within β -case in have been reported to decrease proliferation in murine 72 spleen cells (Bonomi et al., 2011). Studies have also reported that the activity of NF- $\kappa\beta$, one 73 of the major inflammatory signalling pathways, may be reduced in cells exposed to casein 74 hydrolysates (Altmann et al., 2016; Malinowski, Klempt, Clawin-Rädecker, Lorenzen, & 75 Meisel, 2014). The hydrolysis of casein using mammalian, bacterial or plant derived enzymes 76 has also been reported to have immunomodulating effects on Jurkat T cells in other studies 77 (Cermeño et al., 2016; Lahart et al., 2011; Phelan et al., 2009). To the best of our knowledge, 78 no study has compared the cellular antioxidant and anti-inflammatory activities of sodium 79 caseinate hydrolysates generated under the same conditions [hydrolysis time and 80 enzyme:substrate (E:S) ratio], using a range of proteolytic preparations. 81 The aims of the present study were: (i) to generate and examine the physicochemical 82 characteristics of 5 kDa permeates of casein hydrolysates generated using seven different 83 proteolytic preparations, (ii) to compare the antioxidant activity of the hydrolysates in vitro 84 and in H₂O₂-challenged U937 cells; (iii) to determine cytotoxicity in RAW264.7 mouse 85 macrophages, Jurkat T cells and U937 lymphocytes and (iv) to compare the 86 immunomodulatory activity of the 5 kDa permeates of the hydrolysates in RAW264.7 87 88 macrophages and Jurkat T cells.

89

90 2. Materials and methods

91

92 2.1. Materials

| 94 | Human Jurkat T cells, RAW264.7 mouse macrophages and human U937 lymphocytes |
|-----|--|
| 95 | were purchased from the European Collection of Animal Cell cultures (Salisbury, UK). |
| 96 | Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland, UK). Cell |
| 97 | culture plastics were supplied by Cruinn Diagnostics (Dublin, Ireland). Sodium caseinate |
| 98 | (87.57% (w/w) protein) was from Arrabawn Co-op (Tipperary, Ireland). Protease from |
| 99 | <i>Bacillus licheniformis</i> (Alcalase $@2.4L$), protease from <i>Aspergillus oryzae</i> (Flavourzyme $@, \ge$ |
| 100 | 500 U g ⁻¹) and TPCK-trypsin were from Sigma-Aldrich (Wicklow, Ireland). Prolyve 1000 |
| 101 | was from Lyven Enzymes Industrielles (Caen, France) and Flavorpro Whey, Promod 144MG |
| 102 | and Pepsin were all from Biocatalysts (Cefn Coed, Wales, UK). All other cell culture |
| 103 | reagents and chemicals including concanavalin A (ConA), lipopolysaccharide (LPS) and |
| 104 | Trolox were purchased from Sigma-Aldrich, unless otherwise stated. |
| 105 | |
| 106 | 2.2. Generation of casein hydrolysates, determination of the degree of hydrolysis and |
| 107 | ultrafiltration |
| 108 | |
| 109 | Sodium caseinate, reconstituted at 10% (w/v) in distilled water, was equilibrated at 50 |
| 110 | °C with gentle mixing for 1.5 h and the pH was adjusted to pH 7 (or pH 2 for peptic |
| 111 | hydrolysis). Enzymatic hydrolysis was carried out using a pH Stat (718 Stat Titrino, |
| 112 | Metrohm, Herisau, Switzerland) as previously described (Spellman, McEvoy, O'Cuinn, & |
| 113 | FitzGerald, 2003). Hydrolysis was performed using seven different proteolytic preparations |
| 114 | (Alcalase® 2.4L, Prolyve 1000, Flavourzyme®, Flavorpro Whey, Pepsin, TPCK-Trypsin and |
| 115 | Promod 144 MG) at industrially relevant E:S ratios (0.23% for Alcalase® 2.4L, Prolyve 1000 |

| 116 | and Flavourzyme®, Flavorpro Whey and Promod 144 MG, 0.13% for trypsin and 0.25% for |
|-----|--|
| 117 | pepsin) for 4 h, except for the tryptic hydrolysate that was generated over 3 h. |
| 118 | Following hydrolysis, the enzymes were inactivated by heating at 80 °C for 20 min. |
| 119 | Control samples were also included; (i) sodium caseinate was incubated at 50 $^{\circ}$ C without |
| 120 | enzyme and (ii) enzyme was incubated at 50 °C without sodium caseinate. These control |
| 121 | samples were also subjected to heating at 80 °C for 20 min after 4 h incubation. All samples |
| 122 | were freeze-dried (FreeZone 18L, Labconco, Kansas City, USA) and stored at -20 °C until |
| 123 | use. The degree of hydrolysis (DH) of the hydrolysates was determined using the 2,4,6- |
| 124 | trinitrobenzenesulfonic acid (TNBS) method of Adler-Nissen (1979) and as described by Le |
| 125 | Maux, Nongonierma, Barre, and FitzGerald (2016). Ultrafiltration (UF) fractions were |
| 126 | generated by passing the hydrolysate through a membrane having a nominal cut-off of 5 kDa |
| 127 | using a benchtop ultrafiltration system (Sartoflow Alpha, Sartorius AG, Goettingen, |
| 128 | Germany) as described by O'Keeffe and FitzGerald (2014) and were freeze-dried as above. |
| 129 | |
| 130 | 2.3. Reverse phase ultra-performance liquid chromatography and gel permeation high |
| 131 | performance liquid chromatography analysis of UF permeates of casein hydrolysates |
| 132 | |
| 133 | Freeze-dried hydrolysates/ultrafiltration permeates were reconstituted at 1 mg mL ⁻¹ in |
| 134 | mobile phase A [0.1% trifluoroacetic acid (TFA) in MS grade H_2O] and 7 μL was separated |
| 135 | on an ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters, Milford |
| 136 | Massachusetts, USA) at a flow rate of 0.2 μ L min ⁻¹ using an ACQUITY BEH 300 C18 |
| 137 | column (2.1 \times 50 mm, 1.7 µm; Waters, Dublin, Ireland). Mobile phase B was 0.1% TFA in |
| 138 | 80% ACN. Separation was achieved using a linear gradient; 0–0.28 min 100% A; 0.28–45 |

139 min 100–20% A; 45–46 min 20–0% A; 46–48 min 0% A; 48–49 min 0–100% A; 49–51 min

140 100% A. Detector response was measured at 214 nm. Gel permeation high performance

| 141 | liquid chromatography (GP-HPLC) was performed as previously described (Spellman, |
|-----|--|
| 142 | O'Cuinn, & FitzGerald, 2009) with separation achieved through isocratic elution (mobile |
| 143 | phase: 0.1% TFA in 30% ACN at 1.0 mL min ⁻¹) on a TSK G2000 SW column (600×7.5 mm |
| 144 | ID) connected to a TSKGEL SW guard column (75×7.5 mm ID) and the eluent was |
| 145 | monitored at 214 nm. |
| 146 | |
| 147 | 2.4. 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) assay |
| 148 | |
| 149 | The 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) ⁺ (ABTS ⁺) radical |
| 150 | scavenging assay was carried out as described by Re et al. (1999). The ABTS ⁺ radical was |
| 151 | prepared by incubating ABTS solution (7 mM) with potassium persulfate (2.45 mM), an |
| 152 | oxidizing agent, in a ratio of 1.0:0.5 (v/v) at 20 $^{\circ}$ C for 16 h in the dark. The radical was then |
| 153 | diluted using phosphate buffered saline (5 mM, pH 7.4) until an absorbance of 0.70 ± 0.02 at |
| 154 | a wavelength of 734 nm was achieved. Activity was reported based on a standard curve using |
| 155 | Trolox and expressed as µmol Trolox equivalents per gram of freeze-dried powder of |
| 156 | hydrolysate (FDP). The scavenging activity for each sample was determined by three |
| 157 | independent experiments. |
| 158 | |
| 159 | 2.5. Oxygen radical absorbance capacity assay |
| 160 | |
| 161 | The oxygen radical absorbance capacity (ORAC) assay was performed according to |
| 162 | the method of Zulueta, Esteve, and Frígola (2009) with modifications as described by |
| 163 | O'Keeffe and FitzGerald (2014). The final ORAC values were expressed as μ mol of Trolox |
| 164 | equivalents per mg of FDP and were the mean \pm the standard error of three independent |
| 165 | determinations. |

166

167 2.6. *Cell culture*

| 168 | |
|--|--|
| 169 | Jurkat T and U937 cells were maintained in Royal Park Memorial Institute (RPMI) |
| 170 | medium supplemented with 10% (v/v) foetal bovine serum (FBS). RAW264.7 cells were |
| 171 | grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) |
| 172 | FBS. All cell lines were cultured in an atmosphere of CO ₂ -air (5:95, v/v) at 37 °C and were |
| 173 | maintained in the absence of antibiotics. The dried 5 kDa permeates of the casein |
| 174 | hydrolysates were solubilised to a concentration of 10% (w/v) using distilled deionised water, |
| 175 | sterile-filtered using a low protein binding 0.22 μ m Durapore TM millex filter unit (Merck |
| 176 | KGaA) and diluted with sterile DMEM. |
| 177 | |
| 178 | 2.7. Cell proliferation assay |
| 179 | |
| 180 | Jurkat T, U937 and RAW264.7 cells were exposed to increasing concentrations of the |
| 181 | |
| 101 | different 5 kDa permeates (0.05 to 5.0%, w/v) for 24 h in 96 well plates in a final volume of |
| 182 | 200μ L. Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5- |
| 182 183 | different 5 kDa permeates (0.05 to 5.0%, w/v) for 24 h in 96 well plates in a final volume of 200 μ L. Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay (MTT I proliferation kit, Roche Diagnostics; |
| 181 182 183 184 | different 5 kDa permeates (0.05 to 5.0%, w/v) for 24 h in 96 well plates in a final volume of 200 μ L. Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK). |
| 182 183 184 185 | different 5 kDa permeates (0.05 to 5.0%, w/v) for 24 h in 96 well plates in a final volume of 200 μ L. Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK). |
| 182 183 184 185 186 | different 5 kDa permeates (0.05 to 5.0%, w/v) for 24 h in 96 well plates in a final volume of 200 µL. Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK). 2.8. Alkaline single cell gel electrophoresis (comet) assay |
| 182 183 184 185 186 187 | different 5 kDa permeates (0.05 to 5.0%, w/v) for 24 h in 96 well plates in a final volume of 200 µL. Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK). 2.8. Alkaline single cell gel electrophoresis (comet) assay |
| 182 183 184 185 186 187 188 | different 5 kDa permeates (0.05 to 5.0%, w/v) for 24 h in 96 well plates in a final volume of 200 µL. Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK). 2.8. Alkaline single cell gel electrophoresis (comet) assay U937 cells were used to assess the DNA protective effects of the 5 kDa permeates in |
| 182 183 184 185 186 187 188 189 | different 5 kDa permeates (0.05 to 5.0%, w/v) for 24 h in 96 well plates in a final volume of 200 μ L. Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide (MTT) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK). 2.8. Alkaline single cell gel electrophoresis (comet) assay U937 cells were used to assess the DNA protective effects of the 5 kDa permeates in oxidant challenged cells. Cells were seeded at a density of 1 × 10 ⁵ cells mL ⁻¹ and exposed to |

191 μ mol L⁻¹ H₂O₂ for 30 min at 37 °C after which DNA damage was assessed using the comet 192 assay as previously described (Phelan et al., 2009).

193

194 2.9. Cytokine production in Jurkat T and RAW264.7 cells

195

Jurkat T cells were seeded at a density of 2×10^5 cells mL⁻¹ and simultaneously 196 incubated with ConA ($25\mu g mL^{-1}$) and the 5 kDa permeates (0.50 and 0.05%, w/v) for 24 h. 197 Following incubation, the quantity of interleukin (IL)-6), interferon (IFN)-y, IL-2 and IL-10 198 in the media was measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience, 199 Insight Biotechnology Ltd, Wembley, UK). RAW264.7 cells were seeded at a density of 0.2 200 $\times\,10^5$ cells mL $^{-1}$ and simultaneously incubated with LPS (0.1 μg mL $^{-1}$ for IL-6 and tumour 201 necrosis factor (TNF)- α ; 2 µg mL⁻¹ for IL-1 β) and the 5 kDa permeates (0.050% and 0.005, 202 w/v) for 24 h. Following incubation, the content of each of the three cytokines (IL-6, Il-1ß 203 and TNF- α) in the media was measured using ELISA kits. Absorbance was determined at 204 450 nm with a reference wavelength of 570 nm (Varioskan[™] Flash Multimode Reader, 205 Thermoscientific, Waltham, MA, USA). 206

207

209

RAW264.7 cells were seeded at a density of 8×10^5 cells per dish in 60 mm dishes and allowed to adhere overnight. Cells were then stimulated using LPS (0.1 µg mL⁻¹) and treated with the permeates (0.05%, w/v) for 24 h. After this treatment, the RAW264.7 cells were washed using ice cold PBS and lysed using RIPA buffer containing protease (Halt protease inhibitor, Sigma 78439) and phosphatase inhibitors (1 mM NaVO₄, 2.5 mM Na₄O₇P₂ and 2 mM β-glycerophosphate). Cell lysates were then scraped and transferred to Eppendorf

^{208 2.10.} Western blotting

| 216 | tubes and placed on ice for 20 min. Lysates were centrifuged at $25,155 \times g$ for 1 h at 4 °C and |
|-----|---|
| 217 | the supernatant transferred to fresh Eppendorf tubes. Protein concentration was determined |
| 218 | using the BCA method (Smith et al., 1985) and samples were stored at -80 °C until Western |
| 219 | blot analysis. |
| 220 | Proteins were separated using a 10% sodium dodecyl sulphate polyacrylamide |
| 221 | electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene fluoride membrane. The |
| 222 | membrane was then blocked overnight at 4 $^{\circ}$ C using Odyssey Blocking buffer with 0.1% |
| 223 | Tween-20. The following day, the membrane was washed using Tris-buffered saline-Tween- |
| 224 | 20 (TBS-T) and incubated with mouse anti-p65 antibody overnight at 4 °C. Following |
| 225 | incubation, the membrane was again washed using TBS-T and incubated for a further hour at |
| 226 | room temperature with Infrared-labelled goat anti-mouse secondary antibody (LI-COR Inc.). |
| 227 | Finally, the membrane was washed using TBS-T, followed by TBS to remove any remaining |
| 228 | Tween-20. The Odyssey Clx Imager was used to visualise protein bands. Protein loading was |
| 229 | determined using β -actin as a control. |
| 230 | |
| 231 | 2.11. Statistical analysis |
| 232 | |
| 233 | Statistical analysis was determined by ANOVA followed by Dunnett's test or Tukey's |
| 234 | post-hoc test using Prism 5.0 (GraphPad Inc. San Diego, California, USA). Results are |
| 235 | expressed as mean \pm SE or mean \pm SD. Statistical significance was $P < 0.05$. |
| 236 | |
| 237 | 3. Results |
| 238 | |
| 239 | 3.1. Physicochemical characterisation, degree of hydrolysis and in vitro antioxidant |
| 240 | assessment of casein hydrolysates |

| 242 | The different proteolytic preparations produced hydrolysates having distinctly |
|-----|--|
| 243 | different RP-UPLC (Fig. 1) and GPC (Fig. 2) profiles demonstrating the differing |
| 244 | specificities of the proteolytic preparations. The higher specificity of TPCK-Trypsin and |
| 245 | pepsin resulted in a lower proportion of low molecular mass (< 500 Da) peptides present in |
| 246 | these samples (Fig. 2) and in a lower DH (Table 1). Alcalase®, Flavourzyme® and Prolyve |
| 247 | hydrolysis resulted in hydrolysates with the highest proportion of low molecular mass |
| 248 | peptides (< 500 Da) and the highest degree of hydrolysis (~14–18%). |
| 249 | All 5 kDa permeates showed significantly ($P < 0.05$) higher antioxidant activity than |
| 250 | intact sodium caseinate or the full hydrolysates in both the ORAC and ABTS ⁺ assays (data |
| 251 | not shown). There was no significant ($P < 0.05$) difference in antioxidant activity between the |
| 252 | different 5 kDa permeates when measured by the ORAC assay, while the 5 kDa UF permeate |
| 253 | of the Flavourzyme® hydrolysate had the highest activity in the ABTS assay (Table 1); |
| 254 | significantly ($P < 0.05$) higher than that of the 5 kDa UF permeate of the tryptic hydrolysate. |
| 255 | There was no correlation between the DH of the hydrolysates and the antioxidant activity (via |
| 256 | ORAC or ABTS ⁺ assays) of the corresponding 5 kDa UF fractions (Table 1). |
| 257 | |
| 258 | 3.2. Effects of casein hydrolysate 5 kDa permeates on cell proliferation in RAW264.7 |
| 259 | mouse macrophages, human U937 lymphocytes and human Jurkat T cells. |
| 260 | |
| 261 | RAW264.7 mouse macrophages were exposed to increasing concentrations (0–5% |
| 262 | w/v) of the different 5 kDa permeates for 24 h. The MTT assay was then used to assess the |
| 263 | effect of each hydrolysate on cell proliferation. Proliferation was generally unaffected by the |
| 264 | permeates up to a concentration of 1% (w/v) (Table 2). At a concentration of 5% (w/v), cell |
| 265 | proliferation declined significantly ($P < 0.05$) in cells incubated with hydrolysate permeates |

| 266 | generated using Flavorpro Whey, trypsin and pepsin. For Jurkat T cells, the 5 kDa permeates |
|-----|--|
| 267 | obtained from hydrolysates generated using Promod, trypsin and pepsin significantly ($P <$ |
| 268 | 0.05) decreased cell proliferation at 5% (w/v), while the Flavourzyme® hydrolysate permeate |
| 269 | significantly ($P < 0.05$) increased cell proliferation at this concentration (Table 3). A similar |
| 270 | trend was seen with 5 kDa permeates in U937 cells (Table 4). Concentrations of 0.050 and |
| 271 | 0.005% (w/v) were, therefore, selected for bioactivity assays involving RAW264.7 cells. |
| 272 | Non-cytotoxic concentration of 0.50 and 0.05% (w/v) for Jurkat T cells, and 0.05% (w/v) for |
| 273 | U937 were used for bioactivity assays ensuring that all cell viabilities remained greater that |
| 274 | 85%. |
| 275 | |
| 276 | 3.3. Antioxidant activity of casein hydrolysate 5 kDa permeates |
| 277 | |
| 278 | The comet assay was used to assess the DNA protective effect of the 5 kDa permeates |
| 279 | against H ₂ O ₂ -induced DNA damage in U937 cells. Tail DNA was increased to approximately |
| 280 | 80% in U937 cells exposed to H_2O_2 (80 µmol L ⁻¹) for 30 min from a control level of |
| 281 | approximately 16% in untreated cells. Pre-incubation of U937 cells with the different |
| 282 | permeates at 0.05% (w/v) for 24 h did not protect cells against H_2O_2 -induced DNA damage |
| 283 | (Fig. 3). |
| 284 | |
| 285 | 3.4. Cytokine production in Jurkat T cells |
| 286 | |
| 287 | Jurkat T cells were stimulated to produce cytokines using 25 μ g mL ⁻¹ ConA, |
| 288 | following which cytokine (IL-6, IFN- γ , IL-2 and IL-10) production was measured. IL-6 |
| 289 | production was seen to decrease in cells exposed to the 5 kDa permeates (Table 5). All |

hydrolysate permeates at 0.50% (w/v) produced a significant (P < 0.05) decrease in IL-6

production compared with control values. No significant (P < 0.05) effects were seen in IL-2, IL-10 and IFN-γ production after exposure to the 5 kDa permeates at either 0.50 or 0.05% (w/v).

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295 3.5. Cytokine production in RAW264.7 cells

296

RAW264.7 cells were stimulated with LPS at 0.1 μ g mL⁻¹ or 2 μ g mL⁻¹ and treated 297 with 5 kDa permeates for 24 h before cytokine analysis (Table 6). At 0.05% (w/v), each of 298 the hydrolysate fractions caused a significant (P < 0.05) decrease in IL-6 production 299 compared with cells incubated with LPS alone. At 0.005% (w/v), none of the hydrolysate 300 fractions caused significant (P < 0.05) decreases in IL-6 production. Hydrolysate permeates 301 generated using Flavourzyme[®], Flavorpro Whey and trypsin caused a significant (P < 0.05) 302 decrease in IL-1 β production in cells incubated with these samples at 0.050% (w/v). No 303 effect was seen in TNF- α production after treatment with the 5 kDa permeates at any 304 305 concentration.

306

307 3.6. NF-кВ protein expression in RAW264.7 cells

308

RAW264.7 cells were stimulated using 0.1 μ g mL⁻¹ LPS and treated with the 5 kDa permeates at 0.050% (w/v) for 24 h in 60 mm dishes. Cells were then lysed and cell lysates were examined for protein expression of the NF- κ B subunit, p65. All hydrolysate fractions caused a significant (*P* < 0.05) decrease in p65 protein expression compared with cells treated with LPS alone (Fig. 4).

314

315 **4. Discussion**

316

| 317 | It is recognised that utilisation of a variety of analytical techniques is required to |
|-----|--|
| 318 | capture differing pathways of antioxidant activity. The ORAC assay measures the ability of |
| 319 | an antioxidant to prevent peroxyl radical oxidation of a fluorescent probe by means of |
| 320 | hydrogen atom transfer (HAT). In the ABTS assay, the antioxidant present in the permeate |
| 321 | sample scavenges the ABTS radical cation (ABTS $^{\bullet+}$) by means of electron transfer (Power et |
| 322 | al., 2013). In the present study, during initial experimentation it was observed that |
| 323 | concentration of the low molecular mass peptides on processing through a UF membrane |
| 324 | having a molecular mass cut-off of 5 kDa resulted in an increase in the antioxidant activity as |
| 325 | measured by the ORAC assay. However, no further increase in antioxidant activity was |
| 326 | achieved on further processing through a 1 kDa membrane (data not shown). Therefore, the 5 |
| 327 | kDa permeates of each of the hydrolysates were used for all other determinations. |
| 328 | Studies have reported that casein hydrolysates possess non-cellular antioxidant |
| 329 | activity, mainly radical scavenging or electron donating ability. Alcalase® has been |
| 330 | frequently used to produce such hydrolysates (Ao & Li, 2013; Chen & Li, 2012; De Gobba, |
| 331 | Tompa, & Otte, 2014; Xie, Liu, Wang, & Li, 2014; Xie, Wang, Jiang, Liu, & Li, 2015). |
| 332 | Alcalase® was also used in this study; however, no significant difference was seen in the |
| 333 | antioxidant activity of this hydrolysate compared with hydrolysates produced by the other |
| 334 | enzyme preparations and, interestingly, Flavourzyme® produced a hydrolysate with higher |
| 335 | ABTS activity. This may be due to the presence of a larger number of peptides below 500 Da |
| 336 | in the Flavourzyme hydrolysate. |
| 337 | No cellular antioxidant activity was observed with the hydrolysates in the present |

No cellular antioxidant activity was observed with the hydrolysates in the present
study. Previously, Phelan et al. (2009) reported that non-ultrafiltered casein hydrolysates
generated using commercial food-grade enzyme preparations altered glutathione and catalase
(CAT) activity in Jurkat T cells, but did not prevent H₂O₂-induced DNA damage in Caco-2

| 341 | cells. Cermeño et al. (2016) reported that a casein hydrolysate, generated using Prolyve, |
|-----|---|
| 342 | significantly ($P < 0.05$) protected U937 cells from H ₂ O ₂ -induced DNA damage. The 5 kDa |
| 343 | permeate of the Prolyve hydrolysate herein was generated using similar conditions; however, |
| 344 | no cell protective effect was seen. Notably, the full hydrolysate was used in Cermeño et al. |
| 345 | (2016) while our study used the 5 kDa permeate fraction; this may imply that the cell |
| 346 | protective effect was associated with higher molecular mass peptides. Xie et al. (2013) found |
| 347 | that casein hydrolysates produced using Alcalase® or the simulated gastrointestinal digestion |
| 348 | of casein showed significant protective effects in challenged HepG2 cells by reducing oxidant |
| 349 | induced cell death. A follow-on study reported that the Alcalase® hydrolysis of casein |
| 350 | produced hydrolysate fractions which enhanced catalase and superoxide dismutase activity |
| 351 | and increased viability in H ₂ O ₂ -exposed HepG2 cells. The hydrolysate was fractionated based |
| 352 | on charge and negatively charged fractions had greater antioxidant activity (Wang, Xie, & Li, |
| 353 | 2016). Results herein indicate that the enzymes used did not affect antioxidant activity as |
| 354 | hydrolysate 5 kDa permeates with similar activity were produced in all cases. |
| 355 | In the present study, hydrolysates significantly decreased pro-inflammatory cytokine |
| 356 | production (IL-6 and IL-1 β) in T cells and macrophages. Casein hydrolysates, produced using |
| 357 | combinations of TGase and Prolyve were previously reported to significantly decrease IL-6 |
| 358 | production in Jurkat T cells (Cermeño et al., 2016). Malinowski et al. (2014) also reported |
| 359 | that a tryptic hydrolysate of bovine β -casein had significant anti-inflammatory activity in |
| 360 | kidney cells. In this case, casein was hydrolysed for 4 h and the hydrolysate 1–5 kDa |
| 361 | permeate fractions exhibited significant anti-inflammatory activity in human kidney cells. It |
| 362 | was suggested that a group of large hydrophobic peptides were responsible for the anti- |
| 363 | inflammatory activity. The hydrolysis of β -case n using cod tryps in has also been reported to |
| 364 | have anti-inflammatory activity in kidney cells and larger peptides (> 5 kDa) were reported to |
| 365 | have higher activity compared with lower molecular mass peptides (Altmann et al., 2016). |

The hydrolysis of sodium caseinate using a bacterial enzyme has also been reported to
reduce IL-8 production in TNF-α stimulated Caco-2 cells, as well as downregulating several
pro-inflammatory cytokines expression in LPS-stimulated colonic tissue. Activity was
reported to be highest in the 1 kDa retentate fraction in this case (Mukhopadhya et al., 2014).
All the hydrolysates in the present study were 5 kDa permeates and had significant antiinflammatory activity, particularly those produced using trypsin, Flavourzyme® and
Flavorpro Whey.

Two enzyme-only controls were generated based on the high activity of the 373 374 corresponding hydrolysates and these controls were screened for anti-inflammatory activity in RAW264.7 cells to rule out reagent/enzyme related activity. Enzyme-only controls had 375 little anti-inflammatory activity (data not shown), therefore it was determined that the 376 observed anti-inflammatory activity was due to the hydrolysis of casein by these enzymes. 377 Flavourzyme® has previously been shown to generate hydrolysates from brewer's spent 378 grain that were capable of decreasing IFN-y production in Jurkat T cells (McCarthy et al., 379 2013a,b). Bamdad, Shin, Suh, Nimalaratne, and Sunwoo (2017) also reported that a 380 Flavourzyme® generated casein hydrolysate decreased nitric oxide production and TNF-a 381 mRNA expression in RAW264.7 cells; however, this hydrolysate was produced using a 382 combined treatment of high hydrostatic pressure and enzymatic digestion. To the best of our 383 knowledge, no other studies have previously reported anti-inflammatory activity with 384 Flavorpro Whey generated casein hydrolysates. 385

In the present study, while hydrolysate treatment resulted in a decrease in IL-6 and IL-1 β production, TNF- α production was unaffected. Yak milk casein hydrolysates, produced using Alcalase®, were reported to decrease the production of IL-6, IL-1 β and TNF- α in macrophages (Mao, Cheng, Wang, & Wu, 2011). Hydrolysates used in the study by Mao et al. (2011) had significant in vitro antioxidant activity that may have contributed to the

enhanced anti-inflammatory response; IL-6 and IL-1 β production in macrophages was decreased by ~70% and 60%, respectively, in the study by Mao et al. (2011) compared with ~55% and 30%, respectively, in our study. The differing amino acid compositions of milk from different species may also have affected activity, as reported by a study which reported higher antioxidant activity in camel milk casein hydrolysates compared with bovine milk casein hydrolysates (Moslehishad et al., 2013).

The NF- κ B pathway is a major transcription pathway in cells linked to inflammation 397 and chronic inflammatory diseases such as atherosclerosis, inflammatory bowel disease and 398 cancer (Yamamoto & Gaynor, 2001). In the current study, the NF-kB subunit p65 (also 399 known as RELA), which is involved in nuclear translocation and activation, was studied after 400 401 treatment with hydrolysate fractions in LPS-stimulated RAW264.7 cells. The antiinflammatory activity of all seven hydrolysate 5 kDa permeates may be linked to NF-kB 402 activation as the protein expression of p65 was inhibited in all cases. Milk-derived 403 hydrolysates with anti-inflammatory activity have previously been reported to act through the 404 NF-κB pathway in different cell lines (Altmann et al., 2016; Malinowski et al., 2014; 405 Marcone, Haughton, Simpson, Belton, & FitzGerald, 2015; Nielsen, Theil, Larson, & Parup, 406 2012). To the best of our knowledge, our study is the first to examine the molecular 407 mechanism behind the anti-inflammatory activity of bovine casein hydrolysates in 408 macrophages. A study of rice protein hydrolysates, prepared using trypsin, yielded similar 409 results to our study, where IL-6 and IL-1 β expression in LPS-stimulated RAW264.7 cells 410 were decreased after 24 h hydrolysate treatment and p65 translocation to the nucleus was also 411 decreased (Wen et al., 2016). 412

413

414 5. Conclusions

| 416 | The 5 kDa UF permeates of casein hydrolysates produced using mammalian, plant, |
|-----|--|
| 417 | fungal and bacterially-derived proteolytic preparations showed significant in vitro anti- |
| 418 | inflammatory activity in Jurkat T cells and RAW264.7 macrophages. These hydrolysate |
| 419 | permeates had a greater anti-inflammatory effect on IL-6 production in RAW264.7 cells |
| 420 | compared with Jurkat T cells. Three of the hydrolysate permeates also significantly decreased |
| 421 | IL-1 β production in RAW264.7 cells and were produced using either fungal or mammalian |
| 422 | derived enzymes. Based on the ability of the hydrolysate 5 kDa permeates to significantly |
| 423 | inhibit the expression of the NF- κ B subunit, p65, our results also indicate that this anti- |
| 424 | inflammatory activity may be dependent on the NF- κ B inflammatory pathway. Future studies |
| 425 | on the in vivo anti-inflammatory activity of hydrolysate permeates, generated using fungal or |
| 426 | mammalian derived enzymes, may be of interest. |
| 427 | |
| 428 | |
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| 434 | |
| 435 | References |
| 436 | |
| 437 | Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein |
| 438 | hydrolysates by trinitrobenzenesulfonic acid. Journal of Agricultural and Food |
| 439 | Chemistry, 27, 1256–1262. |

| 440 | Altmann, K., Wutkowski, A., Klempt, M., Clawin-Rädecker, I., Meisel, H., & Lorenzen, P. |
|-----|--|
| 441 | C. (2016). Generation and identification of anti-inflammatory peptides from bovine |
| 442 | beta-casein using enzyme preparations from cod and hog. Journal of the Science of |
| 443 | Food and Agriculture, 96, 868–877. |
| 444 | Ao, J., & Li, B. (2013). Stability and antioxidative activities of casein peptide fractions |
| 445 | during simulated gastrointestinal digestion in vitro: Charge properties of peptides |
| 446 | affect digestive stability. Food Research International, 52, 334–341. |
| 447 | Bamdad, F., Shin, S. H., Suh, J. W., Nimalaratne, C., & Sunwoo, H. (2017). Anti- |
| 448 | inflammatory and antioxidant properties of casein hydrolysate produced using high |
| 449 | hydrostatic pressure combined with proteolytic enzymes. Molecules, 22, Article 609. |
| 450 | Bonomi, F., Brandt, R., Favalli, S., Ferranti, P., Fierro, O., Frøkler, H., et al. (2011). |
| 451 | Structural determinants of the immunomodulatory properties of the C-terminal region |
| 452 | of bovine β-casein. International Dairy Journal, 21, 770–776. |
| 453 | Cermeño, M., FitzGerald, R. J., & O'Brien, N. M. (2016). In vitro antioxidant and |
| 454 | immunomodulatory activity of transglutaminase-treated sodium caseinate |
| 455 | hydrolysates. International Dairy Journal, 63, 107-114. |
| 456 | Chakrabarti, S., Jahandideh, F., & Wu, J. (2014). Food-derived bioactive peptides on |
| 457 | inflammation and oxidative stress. BioMed Research International, 2014, Article |
| 458 | 608979. |
| 459 | Chen, M., & Li, B. (2012). The effect of molecular weights on the survivability of casein- |
| 460 | derived antioxidant peptides after the simulated gastrointestinal digestion. Innovative |
| 461 | Food Science and Emerging Technologies, 16, 341–348. |
| 462 | Cheng, X., Gao, DX., Song, JJ., Ren, FZ., & Mao, XY. (2015). Casein |
| 463 | glycomacropeptide hydrolysate exerts cytoprotection against H ₂ O ₂ -induced oxidative |

- 464 stress in RAW 264.7 macrophages via ROS-dependent heme oxygenase-1 expression.
 465 *RSC Advances*, 5, 4511–4523.
- De Gobba, C., Tompa, G., & Otte, J. (2014). Bioactive peptides from caseins released by cold
 active proteolytic enzymes from *Arsukibacterium ikkense*. *Food Chemistry*, *165*, 205–
 215.
- 469 Di Pierro, G., O'Keeffe, M. B., Poyarkov, A., Lomolino, G., & FitzGerald, R. J. (2014).
- Antioxidant activity of bovine casein hydrolysates produced by *Ficus carica* L.derived proteinase. *Food Chemistry*, *156*, 305–311.
- 472 García-Nebot, M. J., Cilla, A., Alegría, A., & Barberá, R. (2011). Caseinophosphopeptides
- 473 exert partial and site-specific cytoprotection against H₂O₂-induced oxidative stress in
 474 Caco-2 cells. *Food Chemistry*, *129*, 1495–1503.
- 475 Kazlauskaite, J., Biziulevicius, G. A., Zukaite, V., Biziuleviciene, G., Miliukiene, V., &

476 Siaurys, A. (2005). Oral tryptic casein hydrolysate enhances phagocytosis by mouse

477 peritoneal and blood phagocytic cells but fails to prevent induced inflammation.

478 International Immunopharmacology, 5, 1936–1944.

- 479 Lahart, N., O'Callaghan, Y., Aherne, S. A., O'Sullivan, D., FitzGerald, R. J., & O'Brien, N.
- 480 M. (2011). Extent of hydrolysis effects on casein hydrolysate bioactivity: Evaluation
 481 using the human Jurkat T cell line. *International Dairy Journal*, *21*, 777–782.
- Le Maux, S., Nongonierma, A. B., Barre, C., & FitzGerald, R. J. (2016). Enzymatic
- 483 generation of whey protein hydrolysates under pH-controlled and non pH-controlled
- 484 conditions: Impact on physicochemical and bioactive properties. *Food Chemistry*,
- 485 *199*, 246–251.
- 486 Malinowski, J., Klempt, M., Clawin-Rädecker, I., Lorenzen, P. C., & Meisel, H. (2014).
- 487 Identification of a NF- κ B inhibitory peptide from tryptic β -casein hydrolysate. *Food*
- 488 *Chemistry*, *165*, 129–133.

| 489 | Mao, XY., Cheng, X., Wang, X., & Wu, SJ. (2011). Free-radical-scavenging and anti- |
|-----|--|
| 490 | inflammatory effect of yak milk casein before and after enzymatic hydrolysis. Food |
| 491 | Chemistry, 126, 484–490. |
| 492 | Marcone, S., Haughton, K., Simpson, P. J., Belton, O., & Fitzgerald, D. J. (2015). Milk- |
| 493 | derived bioactive peptides inhibit human endothelial-monocyte interactions via |
| 494 | PPAR-gamma dependent regulation of NF-kappaB. Journal of Inflammation, 12, |
| 495 | Article 1. |
| 496 | McCarthy, A. L., O'Callaghan, Y. C., Connolly, A., Piggott, C. O., FitzGerald, R. J., & |
| 497 | O'Brien, N. M. (2013a). Brewers' spent grain (BSG) protein hydrolysates decrease |
| 498 | hydrogen peroxide (H ₂ O ₂)-induced oxidative stress and concanavalin-A (con-A) |
| 499 | stimulated IFN-gamma production in cell culture. Food and Function, 4, 1709–1716. |
| 500 | McCarthy, A. L., O'Callaghan, Y. C., Connolly, A., Piggott, C. O., FitzGerald, R. J., & |
| 501 | O'Brien, N. M. (2013b). In vitro antioxidant and anti-inflammatory effects of brewers' |
| 502 | spent grain protein rich isolate and its associated hydrolysates. Food Research |
| 503 | International, 50, 205–212. |
| 504 | Moslehishad, M., Ehsani, M. R., Salami, M., Mirdamadi, S., Ezzatpanah, H., Naslaji, A. N., |
| 505 | et al. (2013). The comparative assessment of ACE-inhibitory and antioxidant |
| 506 | activities of peptide fractions obtained from fermented camel and bovine milk by |
| 507 | Lactobacillus rhamnosus PTCC 1637. International Dairy Journal, 29, 82–87. |
| 508 | Mukhopadhya, A., Noronha, N., Bahar, B., Ryan, M. T., Murray, B. A., Kelly, P. M., et al. |
| 509 | (2014). Anti-inflammatory effects of a casein hydrolysate and its peptide-enriched |
| 510 | fractions on TNFa-challenged Caco-2 cells and LPS-challenged porcine colonic |
| 511 | explants. Food Science and Nutrition, 2, 712–723. |

- 512 Nielsen, D. S., Theil, P. K., Larsen, L. B., & Purup, S. (2012). Effect of milk hydrolysates on
- 513 inflammation markers and drug-induced transcriptional alterations in cell-based

514 models. *Journal of Animal Science*, *90*, 403–405.

- 515 Nongonierma, A. B., O'Keeffe, M. B., & FitzGerald, R. J. (2016). Milk protein hydrolysates
- and bioactive peptides. In P. L. H. McSweeney & J. A. O'Mahony (Eds) *Advanced dairy chemistry* (pp. 417–482). New York, NY, USA: Springer.
- 518 O'Keeffe, M. B., & FitzGerald, R. J. (2014). Antioxidant effects of enzymatic hydrolysates of
 519 whey protein concentrate on cultured human endothelial cells. *International Dairy*
- 520 *Journal*, *36*, 128–135.
- 521 Phelan, M., Aherne-Bruce, S. A., O'Sullivan, D., FitzGerald, R. J., & O'Brien, N. M. (2009).
- 522 Potential bioactive effects of casein hydrolysates on human cultured cells.

523 International Dairy Journal, 19, 279–285,

- 524 Power, O., Jakeman, P., & FitzGerald, R. J. (2013). Antioxidative peptides: enzymatic
- 525 production, in vitro and in vivo antioxidant activity and potential applications of milk-

526 derived antioxidative peptides. *Amino Acids*, 44, 797–820.

- 527 Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999).
- 528 Antioxidant activity applying an improved ABTS radical cation decolorization assay.
- 529 *Free Radical Biology and Medicine*, 26, 1231–1237.
- 530 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M.
- 531 D., et al. (1985). Measurement of protein using bicinchoninic acid. *Analytical*532 *Biochemistry*, 150, 76–85.
- 533 Spellman, D., McEvoy, E., O'Cuinn, G., & FitzGerald, R. J. (2003). Proteinase and
- 534 exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat
- 535 methods for quantification of degree of hydrolysis. *International Dairy Journal, 13*,
- 536 447–453.

| 537 | Spellman, D., O'Cuinn, G., & FitzGerald, R. J. (2009). Bitterness in Bacillus proteinase |
|-----|--|
| 538 | hydrolysates of whey proteins. Food Chemistry, 114, 440-446. |

Tang, W., Yuan, H., Zhang, H., Wang, L., Qian, H., & Qi, X. (2015). An antimicrobial

peptide screened from casein hydrolyzate by *Saccharomyces cerevisiae* cell
membrane affinity method. *Food Control*, *50*, 413–422.

- 542 Trivedi, M., Zhang, Y., Lopez-Toledano, M., Clarke, A., & Deth, R. (2016). Differential
- 543 neurogenic effects of casein-derived opioid peptides on neuronal stem cells:
- 544 implications for redox-based epigenetic changes. *Journal of Nutritional Biochemistry*,
 545 37, 39–46.
- 546 Urista, C. M., Fernández, R. Á., Rodriguez, F. R., Cuenca, A. A., & Jurado, A. T. (2011).
- 547 Review: Production and functionality of active peptides from milk. *Food Science and*548 *Technology International*, *17*, 293–317.
- 549 Wang, B., Xie, N., & Li, B. (2016). Charge properties of peptides derived from casein affect
- their bioavailability and cytoprotection against H₂O₂-induced oxidative stress. *Journal*of Dairy Science, 99, 2468–2479.
- 552 Wen, L., Chen, Y., Zhang, L., Yu, H., Xu, Z., You, H., et al. (2016). Rice protein
- 553 hydrolysates (RPHs) inhibit the LPS-stimulated inflammatory response and
- 554 phagocytosis in RAW264.7 macrophages by regulating the NF-κB signaling pathway.
- 555 *RSC Advances*, *6*, 71295–71304.
- Xie, N., Liu, S., Wang, C., & Li, B. (2014). Stability of casein antioxidant peptide fractions
 during in vitro digestion/Caco-2 cell model: characteristics of the resistant peptides.
- 558 *European Food Research and Technology*, 239, 577–586.
- Xie, N., Wang, B., Jiang, L., Liu, C., & Li, B. (2015). Hydrophobicity exerts different effects
 on bioavailability and stability of antioxidant peptide fractions from casein during

- simulated gastrointestinal digestion and Caco-2 cell absorption. *Food Research International*, 76, 518–526.
- 563 Xie, N., Wang, C., Ao, J., & Li, B. (2013). Non-gastrointestinal-hydrolysis enhances
- bioavailability and antioxidant efficacy of casein as compared with its in vitro
 gastrointestinal digest. *Food Research International*, *51*, 114–122.
- 566 Yamada, A., Sakurai, T., Ochi, D., Mitsuyama, E., Yamauchi, K., & Abe, F. (2015).
- Antihypertensive effect of the bovine casein-derived peptide Met-Lys-Pro. *Food Chemistry*, 172, 441–446.
- 569 Yamamoto, Y., & Gaynor, R. B. (2001). Therapeutic potential of inhibition of the NF-kappa
- 570 B pathway in the treatment of inflammation and cancer. *Journal of Clinical*
- 571 *Investigation*, 107, 135–142.
- 572 Zulueta, A., Esteve, M. J., & Frígola, A. (2009). ORAC and TEAC assays comparison to
- 573 measure the antioxidant capacity of food products. *Food Chemistry*, *114*, 310–316.

1 Figure legends

2

Fig. 1. Reversed phase ultra-performance liquid chromatography profiles of (a) sodium caseinate and
(b-h) casein hydrolysate 5 kDa permeates where the enzymatic preparations employed were: (b)
Alcalase® 2.4L; (c) Flavourzyme®; (d) Prolyve 1000; (e) Flavorpro Whey; (f) Promod 144MG; (g)
Trypsin; (h) Pepsin.

7

Fig. 2. Molecular mass distribution profiles of intact sodium caseinate and 5 kDa ultrafiltration
permeates of sodium caseinate hydrolysates generated with different enzymatic preparations using gel
permeation chromatography; ■, < 500 Da; ■, 1000–500 Da; Ⅲ, 2000–1000 Da; ⊠, 5000–2000 Da;
11 , 10,000–5,000 Da; □, > 10,000 Da.

12

Fig. 3. The ability of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.05%, w/v) to 13 protect against H₂O₂ induced DNA damage in U937 lymphocytes; H, cells treated with H₂O₂ only. 14 15 Tail DNA damage was measured using the comet assay and expressed as a percentage relative to hydrogen peroxide control values (untreated cells). Data are means \pm SD of 2 independent 16 experiments; significance was measured using ANOVA followed by Dunnett's test. 17 18 Fig. 4. Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.05%, w/v) on 19 20 NF-kB (p65) protein expression in RAW264.7 cells relative to cells treated with LPS alone (Control, 21 assigned at 100%); p65 protein expression was assessed in LPS stimulated RAW264.7 cells by Western Blot after 24 h sample treatment. The data show one of three independent experiments, 22 23 which yielded similar results and are the means \pm SE of 3 independent experiments; significance was

24 measured using ANOVA followed by Dunnett's test; * denotes P < 0.05.

Oxygen radical absorbance capacity (ORAC) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activities of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates generated with different enzymatic preparations.^a

| Enzyme | Degree of hydrolysis (%) | ORAC value (µmol TE mg ⁻¹ FDP) | ABTS value (μ mol TE g ⁻¹ FDP) |
|----------------|-----------------------------|--|---|
| Alcalase® | 18.01 ± 1.59^{a} | 1.072 ± 0.07^{a} | $52.86 \pm 3.46^{a,b}$ |
| Flavourzyme® | 14.43 ± 0.61^{b} | 1.130 ± 0.076^{a} | 71.18 ± 7.65^{a} |
| Prolyve | $15.65 \pm 0.60^{a,b}$ | $1.120\pm0.088^{\rm a}$ | $58.38 \pm 3.48^{a,b}$ |
| Flavorpro Whey | 12.86 ± 0.89^{b} | 1.154 ± 0.007^{a} | $67.59 \pm 4.91^{a,b}$ |
| Promod 144MG | $5.23 \pm 0.42^{c,d}$ | 1.080 ± 0.085^{a} | $52.14 \pm 2.19^{a,b}$ |
| Trypsin | $7.21 \pm 0.72^{\circ}$ | 1.080 ± 0.041^{a} | $44.23 \pm 4.83^{b,c}$ |
| Pepsin | 2.31 ± 0.61^{d} | 1.120 ± 0.044^{a} | $59.00 \pm 4.50^{a,b}$ |

^a Abbreviations are: TE, Trolox equivalents; FDP, freeze dried powder of hydrolysate The degree of hydrolysis was calculated for the full hydrolysates prior to ultrafiltration. Data are the mean \pm SEM of 3 independent experiments; values with different superscript letters are significantly different at *P* < 0.05 within each assay.

Table 2

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0-5%, w/v) generated using different enzyme preparations on proliferation in RAW264.7 mouse macrophages.^a

| Enzyme | Cell proliferation (% control) with 5 kDa ultrafiltration permeates (%, w/v) | | | | | | |
|----------------|---|-----------|-----------|-----------|-----------|--|--|
| | 0.05 | 0.10 | 0.50 | 1.00 | 5.00 | | |
| Alcalase® | 109.8 ± 6.7 | 116.8±6.5 | 122.2±7.2 | 120.0±7.6 | 87.0±10.5 | | |
| Flavourzyme® | 106.8±5.8 | 106.7±4.3 | 111.8±5.7 | 110.6±4.6 | 74.0±16.8 | | |
| Prolyve | 107.7±5.5 | 107.7±7.0 | 109.5±5.6 | 109.3±6.1 | 77.8±13.2 | | |
| Flavorpro Whey | 106.7±9.8 | 106.6±5.7 | 103.6±1.2 | 97.9±1.6 | 40.4±9.6* | | |
| Promod 144MG | 99.5±3.1 | 99.2±4.0 | 92.0±9.5 | 90.3±9.3 | 68.9±13.5 | | |
| Trypsin | 68.7±9.5 | 64.0±15.7 | 91.2±5.8 | 89.9±7.7 | 59.2±8.7* | | |
| Pepsin | 107.1±3.5 | 107.1±6.7 | 112.3±5.0 | 110.8±8.2 | 33.6±6.9* | | |

^a RAW264.7 mouse macrophages were seeded at a density of 0.2×10^5 cells mL⁻¹ and treated with increasing concentrations (0–5%, w/v) of samples for 24 h. Cell proliferation was determined using the MTT assay and values are expressed as a percentage relative to untreated cells. Data are the mean \pm SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test; *P* < 0.05) in cell proliferation, compared with untreated RAW264.7 cells.

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0-5%, w/v) on proliferation in human Jurkat T cells. ^a

| Enzyme | Cell proliferation (% control) with 5 kDa ultrafiltration permeates (%, w/v) | | | | | |
|----------------|---|-----------|-----------|------------|-------------|--|
| | 0.05 | 0.10 | 0.50 | 1.00 | 5.00 | |
| Alcalase® | 89.3±7.5 | 84.0±10.4 | 77.9±9.1* | 77.2±8.2* | 84.3±4.3 | |
| Flavourzyme® | 91.1±7.5 | 96.1±1.2 | 96.1±4.0 | 117.6±9.1* | 242.8±12.4* | |
| Prolyve | 96.3±3.8 | 94.1±4.1 | 90.9±6.4 | 94.7±5.1 | 98.6±5.1 | |
| Flavorpro Whey | 95.1±2.8 | 92.7±1.8 | 90.0±1.6* | 101.2±4.8 | 102.0±7.7 | |
| Promod 144MG | 98.3±5.9 | 94.5±5.8 | 81.6±1.3* | 78.8±2.4* | 83.8±4.4* | |
| Trypsin | 96.9±1.7 | 90.7±2.6 | 76.2±9.9* | 79.0±3.6* | 78.4±5.2* | |
| Pepsin | 95.8±7.3 | 94.6±3.4 | 78.9±2.8* | 77.8±2.4* | 13.4±0.5* | |

^a Jurkat T cells were seeded at a density of 1×10^5 cells mL⁻¹ and treated with increasing concentrations (0–5%, w/v) of samples for 24 h. Cell proliferation was determined using the MTT assay and values are expressed as a percentage relative to untreated cells. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test; *P* < 0.05) in cell proliferation, compared with untreated human Jurkat T cells.

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0–5%, w/v) on

proliferation in human U937 lymphocytes. ^a

| Enzyme | Cell proliferation (% control) with 5 kDa ultrafiltration permeates (%, w/v) | | | | | | |
|----------------|---|-----------|-----------|------------|------------|--|--|
| | 0.05 | 0.10 | 0.50 | 1.00 | 5.00 | | |
| Alcalase® | 102.7±3.3 | 96.3±4.3 | 84.9±4.3 | 84.4±4.4 | 76.3±9.8* | | |
| Flavourzyme® | 107.0±7.0 | 101.2±2.0 | 94.6±3.6 | 103.8±10.4 | 159.4±5.3* | | |
| Prolyve | 102.8±3.6 | 97.5±3.5 | 87.5±2.3 | 92.4±4.8 | 91.4±10.4 | | |
| Flavorpro Whey | 95.3±3.1 | 97.6±1.3 | 94.3±1.6 | 94.1±6.8 | 56.8±6.8* | | |
| Promod 144MG | 101.5±6.6 | 89.1±8.3 | 87.6±1.2 | 82.5±5.4 | 71.4±6.3* | | |
| Trypsin | 99.4±5.0 | 94.2±2.7 | 85.8±2.5 | 80.6 ±5.2 | 62.8±9.8* | | |
| Pepsin | 93.7±4.4 | 94.6±1.9 | 83.1±3.9* | 81.2±3.2* | 12.2±0.9* | | |

^a U937 lymphocytes were seeded at a density of 1×10^5 cells mL⁻¹ and treated with increasing concentrations (0–5%, w/v) of samples for 24 h. Cell proliferation was determined using the MTT assay and values are expressed as a percentage relative to untreated cells. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test; *P* < 0.05) in cell proliferation, compared with untreated human U937 cells.

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.50 and 0.05%, w/v) on IL-6, IFN- γ , IL-2 and IL-10 production in Jurkat T lymphocytes. ^a

| Sample | Cytokine pro | duction (% contro | ol) | | | | | |
|----------------|--------------|-------------------|-----------|-----------|------------------|-----------|-----------------|-----------|
| | IL-6 | | IFN-γ | | IL-2 | | IL-10 | |
| | 0.50% | 0.05% | 0.50% | 0.05% | 0.50% | 0.05% | 0.50% | 0.05% |
| Control | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | $100.0 \pm .0.0$ | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
| Alcalase® | 76.7±2.8* | 87.8±2.9 | 91.0±3.9 | 97.5±2.8 | 103.4±3.8 | 103.4±3.8 | 105.9 ± 4.0 | 103.8±2.9 |
| Flavourzyme® | 81.7±2.1* | 87.9±2.1 | 89.1±3.9 | 94.2±2.4 | 101.7±4.7 | 101.7±4.7 | 111.2±3.3 | 104.2±5.0 |
| Prolyve | 79.5±1.0* | 92.8±4.1 | 94.2±3.2 | 100.3±1.5 | 95.0±5.9 | 95.0±5.9 | 102.7 ± 5.1 | 105.2±0.4 |
| Flavorpro Whey | 83.6±0.8* | 92.0±2.0 | 103.6±3.6 | 100.7±5.2 | 103.3±5.9 | 103.3±5.9 | 106.9±3.8 | 107.2±4.8 |
| Promod 144MG | 79.4±1.5* | 99.9±3.2 | 93.5±2.6 | 103.8±3.6 | 100.9±3.3 | 100.9±3.3 | 111.2±7.6 | 108.1±6.3 |
| Trypsin | 80.9±1.5* | 102.7±4.1 | 88.8±2.7 | 105.7±3.5 | 116.7±8.4 | 116.7±8.4 | 116.0±6.1 | 104.3±9.3 |
| Pepsin | 87.2±2.1* | 102.2±2.6 | 97.2±1.4 | 110.0±7.6 | 98.2±3.2 | 98.2±3.2 | 102.1±5.0 | 99.6±1.2 |

^a Jurkat T cells were seeded at a density of 2×10^5 cells mL⁻¹, stimulated with ConA (25 µg mL⁻¹) and treated with 0.50 and 0.05% (w/v) 5 kDa UF permeates of sodium caseinate hydrolysates for 24 h. Cytokine production was measured using the enzyme-linked immunosorbent assay (ELISA) and values were expressed as a percentage relative to cells treated with ConA alone (control): IL-6 concentration, control = 0.004 ng mL⁻¹; IFN- γ concentration, control = 0.099 ng mL⁻¹; IL-2 concentration, control = 0.147 ng mL⁻¹; IL-10 concentration, control = 0.156 ng mL⁻¹. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test; *P* < 0.05) in cytokine production, compared with Jurkat T cells treated with ConA alone.

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.050 and 0.005%, w/v) on IL-6, IL-1 β and TNF- α production in RAW264.7 mouse macrophages.^a

| | | | | | | \mathbf{N} |
|----------------|----------------|--------------------|-----------|------------|------------------|--------------|
| Sample | Cytokine produ | uction (% control) | | | | |
| | IL-6 | | IL-1β | | TNF-α | |
| | 0.050% | 0.005% | 0.050% | 0.005% | 0.050% | 0.005% |
| Control | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 | 100.0±0.0 |
| Alcalase® | 55.4±7.4* | 100.7 ± 21.1 | 78.8±5.3 | 118.4±7.8 | 109.8±5.1 | 114.5±5.2 |
| Flavourzyme® | 55.0±12.4* | 82.1±7.1 | 74.7±8.2* | 121.4±9.3 | 109.6±4.7 | 110.4±3.9 |
| Prolyve | 54.2±3.8* | 87.2±13.3 | 77.6±4.1 | 107.4±13.9 | 104.3±4.8 | 110.6±3.3 |
| Flavorpro Whey | 47.2±8.9* | 79.5±4.6 | 76.4±3.7* | 113.2±14.5 | 107.5±5.1 | 112.4±4.7 |
| Promod 144MG | 58.4±12.0* | 87.9±8.1 | 96.1±2.9 | 115.0±13.0 | 108.1±5.6 | 109.4±4.0 |
| Trypsin | 60.3±9.8* | 99.8±19.7 | 74.8±2.1* | 105.1±16.7 | 112.4±6.0 | 114.0±4.9 |
| Pepsin | 55.7±4.0* | 79.0±9.7 | 86.2±7.2 | 111.0±15.6 | $102.0{\pm}10.8$ | 104.7±3.4 |

^a RAW264.7 mouse macrophages were seeded at a density of 0.2×10^5 cells mL⁻¹, stimulated with LPS (0.1 µg mL⁻¹ or 2µg mL⁻¹) and treated with 0.050 and 0.005% (w/v) 5 kDa UF permeates of sodium caseinate hydrolysates for 24 h. Cytokine production was measured using the enzyme-linked immunosorbent assay (ELISA) and values were expressed as a percentage relative to cells treated with LPS alone (Control): IL-6 concentration, control = 0.219 ng mL⁻¹; IL-1 β concentration, control = 0.180 ng mL⁻¹; TNF- α concentration, control = 0.721 ng mL⁻¹. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test; *P* < 0.05) in cytokine production, compared with RAW264.7 mouse macrophages treated with LPS alone.

A CONTRACTION MANUSCONTRA

A CONTRACTION MANUSCONTRA



Figure 1:





ACCEPTED MANUSCRIPT

