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1	In vitro antioxidant and immunomodulatory activity of transglutaminase-treated
2	sodium caseinate hydrolysates.
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27 ABSTRACT

29	Sodium caseinate (NaCN) was incubated prior to and after hydrolysis with a microbial
30	transglutaminase (TGase) and hydrolysed with Prolyve 1000. The resultant hydrolysates were
31	tested for their immunomodulatory and antioxidant activity. TGase-treated hydrolysates
32	significantly reduced ($p < 0.05$) the production of IL-6 at 0.5 and 1 mg mL ⁻¹ and the non-
33	TGase treated hydrolysate reduced the production of IL-6 at 1 mg mL ⁻¹ in concanavalin
34	(ConA) stimulated Jurkat T cells. None of the samples had an effect on IL-2. The
35	hydrolysates showed higher oxygen radical absorbance capacity assay and ferric reducing
36	antioxidant power activity than unhydrolysed NaCN, but no significant ($p > 0.05$) differences
37	were found between the TGase-treated and non-TGase-treated samples. In the presence of
38	hydrogen peroxide, the non-TGase-treated sample exhibited the highest DNA protective
39	effect in U937 cells. These findings suggest that NaCN derived hydrolysates with and
40	without treatment with TGase may exert specific antioxidant, genoprotective and anti-
41	inflammatory effects.
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51 1. Introduction

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53 Approximately 30% of occidental population deaths are due to diseases related to cardiovascular problems (WHO, 2011). The continuous exposure to chemicals, unhealthy 54 diets and sedentary life-style may be contributing factors for premature ageing and illness. 55 Oxidative stress is an imbalance between the production of free radicals or reactive oxygen 56 species (ROS) and the neutralisation of these by antioxidant compounds (Lobo, Patil, Phatak, 57 & Chandra, 2010). The excess of ROS produced as a result of oxidative stress is involved in 58 59 the pathogenesis of neurodegenerative, cardiovascular and inflammatory diseases. For instance, atherosclerotic cardiovascular disease is characterised by the oxidation of low-60 density lipoproteins (LDL) which induce the adhesion and influx of monocytes and lead to 61 62 cytokine production, a pro-inflammatory response (Singh, Devaraj, & Jialal, 2005). Some multifactorial diseases such as atherosclerosis or Parkinson's disease are the result of 63 combined inflammatory and oxidative processes (Chen, Lü, Yao, & Chen, 2016). For this 64 65 reason, there is an increasing interest in studies on the anti-inflammatory and antioxidant potential of bioactive dietary ingredients. 66 Bioactive peptides (BAPs) are natural protein fragments obtained from food proteins 67 such as dairy, eggs, fish, meat or vegetables. These peptides can be released from proteins by 68 bacterial fermentation, digestion or enzymatic hydrolysis and they may possess potent 69 70 bioactivities (Korhonen & Pihlanto, 2006; Nongonierma, O'Keeffe, & FitzGerald, 2016). Antioxidant BAPs may inhibit the action of free radicals, reducing oxidation events and 71 thereby contribute to the prevention of inflammatory responses. Antioxidant and anti-72 73 inflammatory bioactivities are directly related (Pashkow, 2011). Caseins from bovine milk contain a large number of bioactive peptides encrypted into the parent protein (Hernández-74 Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014; Nongonierma & 75

FitzGerald, 2015; Nongonierma et al., 2016; Phelan, Aherne, FitzGerald, & O'Brien, 2009a;
Power, Jakeman, & FitzGerald, 2012; Wada & Lönnerdal, 2014). The composition, structure,
hydrophobicity, position of amino acid residue and molecular mass are factors directly
related with the activity of BAPs (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara,
1998). The amino acid composition of casein, which is rich in Pro residues, makes it a
potential source of bioactive peptides for the production of biofunctional foods (Pihlanto,
2006).

Enzymatic hydrolysis of casein proteins has resulted in the generation of BAPs with 83 84 demonstrated immunomodulatory and antioxidant activities. Two casein hydrolysates, deriving from digestion with Lb. helveticus MIMLh5 and Lb. acidophilus ATCC 4356 85 proteinases, demonstrated anti-inflammatory activity by decreasing NF-κB activity in 86 recombinant Caco-2 cells (Stuknyte, De Noni, Guglielmetti, Minuzzo, & Mora, 2011). A 87 recent study demonstrated that a <5 kDa NaCN hydrolysate was able to reduce IL-8, a pro-88 inflammatory cytokine, in tumour necrosis factor-alpha (TNF-α) treated Caco-2 cells, and 89 90 similar results were observed ex vivo in porcine colonic tissue (Mukhopadhya et al., 2015). Similarly, a peptide obtained from β -CN (f 94-98), QEPVL, and its derivative, QEPV, 91 showed the capacity to regulate the inflammatory process not only in vitro but also in vivo in 92 Balb/c mice (Jiehui et al., 2014). Studies using Balb/c mice reported that yak casein 93 hydrolysates possessed radical scavenging activities against 2,2-Diphenyl-1-picrylhydrazyl 94 (DPPH), superoxide and hydrogen peroxide, and also decreased the production of nitric oxide 95 (NO) and the pro-inflammatory IL-6 and IL-1β cytokines (Mao, Cheng, Wang, & Wu, 2011). 96 The antioxidant properties of casein hydrolysates have been widely reviewed (Pihlanto, 2006; 97 98 Power et al., 2012).

99 Furthermore, the combination of cross-linking and enzymatic hydrolysis in casein
100 may lead to the generation of novel peptides with new bioactivities due to the intra and inter

101 cross-links created within the casein peptide structure. Cross-linking with TGase is known to improve the physicochemical and organoleptic properties of dairy products. The addition of 102 TGase improved the emulsifying and foaming properties of NaCN (Flanagan & FitzGerald, 103 2003). The application of TGase in yoghurt and cheese is well established leading to 104 improved product quality (Özer, Hayaloglu, Yaman, Gürsoy, & Şener, 2013; Romeih, Abdel-105 Hamid, & Awad, 2014). However, little is known about the effect of TGase on the bioactivity 106 of peptides. A recent study by Hong, Gottardi, Ndagijimana, and Betti (2014) found that 107 glycopeptides from fish, obtained by glycosylation and proteolytic hydrolysis with Alcalase, 108 improved their cellular antioxidant activity in HepG2 cells and their lipid oxidation inhibition 109 activity with the addition of TGase. Additionally, gluten hydrolysates glycosylated with 110 TGase, have been reported to improve their in vitro antioxidant activity (Gottardi, Hong, 111 Ndagijimana, & Betti, 2014). Preliminary work in our laboratory has shown that samples 112 treated with TGase prior to hydrolysis had an anti-inflammatory activity in LPS induced 113 Jurkat T cells; however, no antioxidant activity was detected (O'Sullivan, Lahart, 114 O'Callaghan, O'Brien, & FitzGerald, 2013). 115 The aim of the present study was to assess the effect of enzymatic hydrolysis and its 116 combination with TGase cross-linking treatment on the immunomodulatory and antioxidant 117 activity of NaCN hydrolysates. 118 119 120 2. Materials and methods 121 2.1. **Materials** 122 123 Sodium caseinate (NaCN; 87.57%, (w/w, protein) was provided by Arrabawn Co-op 124 Society Ltd., Tipperary, Ireland. Calcium independent TGase from *Streptoverticillium* spp. 125

126	was provided by Forum Products Ltd. (Brighton Rd., Redhill, Surrey, UK). Prolyve 1000™
127	was kindly provided by Lyven Enzymes Industrielles (Caen, France). U937 and Jurkat T
128	cells were obtained from the European Collection of Animal Cell Cultures (ECACC,
129	Salisbury, Wilts, UK). MTT I proliferation kit was obtained from Roche Diagnostics
130	(Burgess Hill, West Sussex, UK). IL-6 and IL-2 eBioscience enzyme-linked
131	immunoadsorbent assay (ELISA) Ready-SET-Go kits were purchased from Insight
132	Biotechnology (Wembley, UK). All other chemicals and reagents were purchased from
133	Sigma Chemical Company Ltd. (Wicklow, Ireland), unless otherwise stated.
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135	2.2. Generation of cross-linked NaCN hydrolysates
136	
137	TGase-treated hydrolysates were generated prior to (TGase/Prolyve) and after
138	(Prolyve/TGase) Prolyve hydrolysis. For the generation of the Prolyve/TGase hydrolysate
139	sample a NaCN solution (10%, w/v) was incubated with 0.3% (v/v) Prolyve 1000 TM at 50 °C
140	and pH 7 using a pH stat (Titrando 843, Metrohm, Dublin, Ireland). After 240 min of
141	incubation, the enzymatic reaction was stopped by heating at 80 °C for 20 min. An aliquot of
142	this solution was used as a non-TGase-treated hydrolysate (Prolyve). Then the resultant
143	solution was incubated with TGase (2%, v/v) at room temperature and pH 7.0 for 180 min.
144	Inactivation of TGase was carried out by heating at 80 °C for 20 min. For the generation of
145	the TGase/Prolyve hydrolysate sample, NaCN was incubated firstly with TGase (2%, v/v)
146	and subsequently submitted to hydrolysis with Prolyve 1000 TM using the same conditions as
147	outlined above. All the hydrolysates generated where further subjected to in vitro digestion
148	with pepsin (enzyme:substrate ratio 1:40, w/w) for 90 min at 37 °C at pH 2.0 and
149	subsequently with Corolase $PP^{\text{(B)}}$ (enzyme:substrate ratio 1:10, w/w) for 180 min at 37 °C at
150	pH 7 to simulate in vitro gastrointestinal digestion (SGID; Walsh et al., 2004).

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152 2.3. Cell culture

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154	A leukaemic monocytic lymphoma cell line, U937 cells, and a human leukaemic T
155	cell line, Jurkat T cells, were maintained at 37 °C in a 5% CO ₂ atmosphere, in antibiotic-free
156	medium (RPMI-1640) supplemented with 10% foetal bovine serum (FBS). Reduced serum
157	media (2.5% FBS) was used during experiments.
158	
159	2.4. Cell viability assay
160	
161	Cells at a density of 1×10^5 cells mL ⁻¹ in growth media were seeded in each well of 96-
162	well flat-bottom plates. Cells were incubated with hydrolysates (0–50 mg mL ⁻¹) at 37 °C for
163	24 h. Following incubation, cell viability was determined using the MTT [3-(4,5-
164	dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. Briefly, media was removed
165	(100 μ L) and MTT reagent 1 (5 μ L) was added and cells were incubated for 4 h at 37 °C.
166	Then, MTT reagent 2 (100 μ L) was added to the cells and the plate was incubated overnight
167	at 37 °C. The absorbance was measured on a Varioskan Flash microplate reader
168	(ThermoScientific, Tewksbury, MA, USA) at 570 nm. The half maximal effective
169	concentration (EC ₅₀) values were calculated in triplicate ($n = 3$) and expressed as mg mL ⁻¹
170	using GraphPad Prism 4.
171	
172	2.5. Immunomodulatory activity – cytokine production
173	
174	Jurkat T cells, at a density of 2×10^5 cells mL ⁻¹ , were seeded in 96-well plates in the
175	presence of concanavalin A (ConA, 50 μ g L ⁻¹) and were incubated with test samples at 0.5

and 1 mg mL⁻¹ for 24 h at 37 °C. Production of the cytokines IL-6 and IL-2 was determined using ELISA kits. Absorbance was read at 450 nm using a microplate reader. Experiments were performed in triplicate (n = 3) and data were expressed as a percentage of the stimulated cell control.

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181 2.6. Antioxidant activity

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183 2.6.1. Intracellular reduced glutathione (GSH)

U937 cells (1×10^5 cells mL⁻¹, 5mL) were incubated with NaCN and its hydrolysates 184 (0.5%, v/v) in a 96 well plate for 24 h at 37 °C. Following incubation, cells were harvested, 185 sonicated on ice at 13 mA for 30 s, centrifuged $(14,000 \times g, 30 \text{ min}, 4 \text{ °C})$ and the supernatant 186 was collected. An aliquot (100 µL) of sample was mixed with 0.01 M sodium phosphate-187 0.005 M ethylenediamine tetraacetic acid buffer (1.8 mL) and o-phthaldialdehyde (0.1 mg). 188 The fluorescence was determined at 350 nm (absorption) and 420 nm (emission). The GSH 189 content was determined from a standard curve using known concentrations of GSH and the 190 results were expressed relative to the protein content. The protein content of the samples was 191 determined by the bicinchoninic acid (BCA) protein assay as previously described by Smith 192 et al. (1985). The assay was performed in triplicate (n = 3). 193

194

195 *2.6.2. Comet assay*

196 U937 cells $(1 \times 10^5 \text{ cells mL}^{-1})$ were treated with 5 mg mL⁻¹ (0.5%, v/v) of test sample 197 for 24 h in a 6-well plate (final volume 2 mL) at 37 °C. After incubation, cells were treated 198 with 50 μ M H₂O₂ or 400 μ M tert-butyl hydroperoxide (t-BOOH) for 30 min. The comet assay, 199 previously described by McCarthy et al. (2012), was then used to measure oxidative DNA 200 damage. Cells were harvested and transferred to microscope slides (prepared with normal

201	gelling agarose; NGA) and covered with low melting point agarose (LMP). The slides were
202	placed in lysis solution for 1 h at 4 $^{\circ}$ C, followed by electrophoresis at 300 mA, 20 V for 25
203	min. The slides were then neutralised using 0.4 M Tris-base at pH 7.5 and stained with
204	ethidium bromide (20 μ g mL ⁻¹). Cells were visualised under a fluorescence microscope and
205	Komet 5.5 image analysis software was used to score 50 cells per slide. The DNA damage
206	was performed in quadruplicate $(n = 4)$ and expressed as percentage of tail DNA.

207

208 2.6.3. Oxygen radical absorbance capacity assay

209 The oxygen radical absorbance capacity (ORAC) assay was performed as described by Zulueta, Esteve, and Frígola (2009) using a Synergy[™] HT plate reader (BioTek 210 Instruments Limited, Bedfordshire, UK). An aliquot (50 µL) of test sample (0.1 mg mL⁻¹), 211 212 standard or phosphate buffer (75 mM) and 50 µL of fluorescein (0.78 µM) were added into a microtitre plate incubated at 37 °C. The reaction was started with the addition of 25 µL of 213 2.2'-azobis-2-methyl-propanimidamide (AAPH) to each well. Fluorescence readings were 214 recorded every 5 min for 120 min at excitation and emission wavelengths of 485 and 520 nm, 215 respectively. The ORAC values, expressed as µmoles trolox equivalents (TE) per mg freeze 216 dried sample, were calculated using trolox as a standard. Experiments were performed in 217 triplicate (n = 3). 218

219

220 2.6.4. Ferric reducing antioxidant power activity

The ferric reducing antioxidant power (FRAP) value of hydrolysate samples was
determined using the method described by Benzie and Strain (1999) with some
modifications. Briefly, 2 mL of freshly prepared FRAP reagent [150 μL; 0.3 M acetate buffer
(pH 3.6), 0.01 M 2,4,6-tripyridyl-s-triazine (TPTZ), 0.02 M FeCl₃·6H₂O 10:1:1] heated to
37 °C was added into a cuvette and the absorbance was read at 590 nm. Test sample

226	(100 μ L), FeSO ₄ (standard) and MeOH (blank) was then added and the absorbance (590 nm)
227	was read after 30 min incubation at 37 °C. The experiment was performed in triplicate (n =
228	3) and the FRAP values (μ M) were calculated from the standard curve.
229	
230	2.6.5. 2.2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonate) radical scavenging assay

The 2.2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS+) radical scavenging 231 232 activities were measured using the previously described method by Re et al. (1999). The ABTS++ solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulphate 233 234 for 16 h to generate the radicals. The radical solution was then diluted to an absorbance of 0.70 ± 0.02 at 734 nm. Test samples (10 µL) at a final concentration 1 mg mL⁻¹ were added in 235 a 96 well plate with the radical solution (200 µL) and kept in the dark at room temperature 236 237 for 5 min. Absorbance was then measured at 734 nm. Known concentrations of trolox solutions were used to plot a standard curve and ABTS+ values were calculated. All samples 238 were analysed in triplicate (n=3) and the final inhibitory activity was expressed as %ABTS 239 inhibition using the following equation: 240

241

ABTS inhibitory activity (%) = $[(Abs_{blank} - Abs_{sample}) / Abs_{blank}] \times 100$

242

243 2.6.6. DPPH radical scavenging assay

The DPPH assay was carried out according to the method described by Brand-Williams, Cuvelier, and Berset (1995). Concentrations of trolox ranging from 0.04 to 0.40 μ M were used to prepare a standard curve for calibration. Hydrolysate test samples (100 μ L) at a final concentration 1 mg mL⁻¹ were diluted with methanol and incubated with 3.9 mL of 6 μ M DPPH reagent for 30 min. Absorbance was read at 515 nm at 0 and 30 min. All samples were analysed in triplicate (n=3) and the results were expressed as % DPPH inhibition using the following equation:

251		DPPH inhibition (%) = [(Abs _{blank} - Abs _{sample}) / Abs _{blank}] \times 100
252		
253	2.7.	Statistical analysis
254		
255		All data were determined as the mean and standard error values of at least three
256	indep	endent experiments. Data were analysed by one-way analysis of variance (ANOVA)
257	follov	ved by Dunnett's test (or Tukey's multiple comparison test, where appropriate), using
258	Graph	n-Pad Prism 4 (Graph-Pad software, California, U.S.A.).
259		
260	3.	Results and discussion
261		
262	3.1.	Effect of casein hydrolysates on cell viability
263		
264		Cell viability of Jurkat T (Table 1) and U937 (Table 2) cells was measured by the
265	MTT	assay to determine non-toxic concentrations of hydrolysates to be used for subsequent
266	exper	iments. The MTT assay measures cellular mitochondrial activity by assessing the
267	activi	ty of mitochondrial reductase. Cells were incubated with increasing concentrations of
268	NaCN	I and its hydrolysates (0–5%, v/v, equivalent to 0–50 mg mL ⁻¹). The EC ₅₀ values were
269	calcul	ated and represent the concentration required that inhibits cell viability by 50%. Based
270	on the	EC_{50} values obtained, the hydrolysates seemed to have similar cytotoxic effects on
271	Jurka	t T and U937 cells (Table 3). McCarthy et al. (2013) reported the cytotoxic effect of
272	brewe	ers' spent grain (BSG) hydrolysates on U937 cells was higher than on Jurkat T cells. In
273	the pr	esent study, samples at 0.5% (v/v) showed significant ($p < 0.05$) inhibition of the
274	viabil	ity of Jurkat T cells compared with control (non-treated cells) supporting the previous
275	result	s from Lahart et al. (2011). Lahart et al. (2011) reported that a NaCN hydrolysate at

276 0.25% (v/v), obtained with Alcalase (A4), decreased the viability of Jurkat T cells to 61.2% compared with untreated cells (100%). All the hydrolysates studied herein induced a 277 significant (p < 0.05) cytotoxic activity at concentrations of 0.5% (v/v) in Jurkat T cells. In a 278 study by Phelan, Aherne-Bruce, O'Sullivan, FitzGerald, and O'Brien (2009b), NaCN 279 hydrolysates prepared using different food-grade enzyme preparations were cytotoxic to 280 Jurkat T cells at a concentration of 0.5% (v/v). The addition of 100 μ g mL⁻¹ of a 281 glycomacropeptide from bovine milk was reported to significantly inhibit the viability of 282 U937 cells (Li & Mine, 2004). In contrast, in our study concentrations of the hydrolysates up 283 to 5 mg mL⁻¹ (corresponding to 0.5%, v/v), showed no inhibition in the viability of U937 284 cells. The conformation, degree of hydrolysis and the source of the proteolytic enzyme used 285 to generate the hydrolysates are key factors that may affect the cytotoxicity of hydrolysates 286 (Lahart et al., 2011; Zou, He, Li, Tang, & Xia, 2016). TGase treated hydrolysates showed 287 similar results in Jurkat T cells to those reported by O'Sullivan et al. (2013). Sample 288 concentrations that showed a cell viability < 75% of control were considered toxic. 289 Therefore, non-toxic test sample concentrations of 0.5 and 1 mg mL⁻¹ were used for 290 subsequent immunomodulatory and antioxidant activities in both cell lines. 291

292

293 3.2. Immunomodulatory effects of NaCN hydrolysates

294

The anti-inflammatory activity of intact NaCN, the NaCN hydrolysate (Prolyve) and the cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase) was screening by measuring their potential to supress the production of IL-2 and IL-6 in ConA stimulated Jurkat T cells. ConA is a lectin mitogen known for its ability to stimulate the T-cell receptor and the subsequent activation of signalling pathways involving nuclear factor of activated Tcells (NFAT) and mitogen-activated protein kinase (MAPK) pathways resulting in the

301	production of cytokines (Takahashi et al., 2009; Tanaka, Akaishi, Hosaka, Okamura, &
302	Kubohara, 2005). The results showed that all hydrolysates tested significantly reduced ($p < p$
303	0.05) IL-6 production (Table 4). This effect was dose dependent. IL-6 production was
304	significantly decreased ($p < 0.05$) by NaCN up to 41.85 and 30.21% of the control ConA-
305	stimulated cells at concentrations of 0.5 and 1 mg mL ⁻¹ , respectively. In contrast, a study
306	using yak casein showed that intact casein did not produce a decrease in IL-6, whereas its
307	hydrolysates decrease cytokine production in LPS-stimulated macrophages (Mao et al.,
308	2011). The production of cytokines in LPS-induced RAW cells incubated with yak casein
309	hydrolysates has been previously reported by Mao et al. (2011). The study reported that at a
310	concentration of 0.5 mg mL ⁻¹ the hydrolysates significantly inhibited the production of the
311	pro-inflammatory cytokines IL-6, IL-1 β and TNF- α . A recent study showed similar results in
312	ConA induced Jurkat T cells incubated with NaCN hydrolysates whereby IL-6 cytokine
313	production was significantly decreased compared with the control whereas IL-2 production
314	was unchanged (O'Sullivan, O'Callaghan, O'Keeffe, FitzGerald, & O'Brien, 2015). In an
315	earlier study, Phelan et al. (2009b) studied the effect of eight distinct casein hydrolysates
316	generated with several food-grade enzyme preparations on IL-2 production in Con-A induced
317	Jurkat T. The study demonstrated that six of the hydrolysates enhanced the secretion of IL-2.
318	The authors suggest that this pro-inflammatory effect might be useful on regulation of
319	deficient immune processes. Yak casein hydrolysates were also reported to increase IL-2
320	production in ConA stimulated spleen cells (Mao, Yang, Song, Li, & Ren, 2007).
321	Nevertheless, in the present study, NaCN and its hydrolysates did not have any effect on
322	ConA stimulated IL-2 production in Jurkat T cells. NaCN at 1 mg mL ⁻¹ significantly reduced
323	the production of IL-2 (79%) compared with the control (Table 4). Similarly, Lahart et al.
324	(2011) found no difference in the secretion of IL-2 in Jurkat T cells incubated with 0.5%
325	(v/v) of intact NaCN or 0.5% (v/v) of NaCN hydrolysates generated with Alcalase and

326 Flavourzyme. However, O'Sullivan et al. (2013) reported a decrease in IL-2 production in ConA-stimulated Jurkat T cells incubated with NaCN cross-linked with TGase pre-327 hydrolysis. The extent of hydrolysis reached and the enzymatic preparation used to generate 328 329 the hydrolysates are mainly responsible for the final sequences of peptides within the hydrolysates and could induce different cell reactions. It is interesting to note a study where 330 the substitution of proline in short peptides had a negative effect on their immunomodulatory 331 activity, but the substitution of proline with proline analogues did not have an impact on the 332 final bioactivity. The study reported on an immunomodulatory peptide from β -casein (191– 333 209) (LLYQEPVLGPVRGPFPIIV) which was synthesised with modifications around Pro 334 residues. In particular, substitution of the last proline (P206) with D-Pro produced an 335 inhibition in the in vitro immunosuppressory effects in α -CD3 and α -CD28 stimulated murine 336 spleen cells (Bonomi et al., 2011). Hence, the structure and sequence of peptides is a crucial 337 factor which directly affects their anti-inflammatory activity. 338

- 339
- 340 3.3. Cellular antioxidant assays
- 341

Reduced GSH, an important antioxidant, is produced within the cells to prevent cell 342 damage induced by ROS. Incubation with the NaCN hydrolysates led to a small increase in 343 GSH concentration in U937 cells, whereas the parent protein, unhydrolysed NaCN, produced 344 a reduction of the GSH content (Table 5). However, none of the results was statistically 345 significant compared with the untreated cells. These results are in agreement with those 346 reported by O'Sullivan et al. (2013) where no difference in GSH content was found in 347 TGase-treated NaCN hydrolysates in Jurkat T cells. In contrast, GSH content and catalase 348 activity were increased by NaCN hydrolysates in Jurkat T cells (Lahart et al., 2011; Phelan et 349 al., 2009b). Some studies suggest that the peptide profile affects its antioxidant activity. For 350

351 instance, peptides from whey protein hydrolysates had a more effective protecting ability against oxidative stress in PC12 cells as their hydrophobicity increased (Zhang et al., 2015). 352 The hydrophobicity of the peptide residues enhances the accessibility of the peptide to the 353 fatty acids in cell membranes, which are subjected to oxidation by free radicals and ROS 354 (Aluko, 2012). The proteolytic enzyme used to obtain the hydrolysates is another key factor 355 in hydrolysate bioactivity. For instance, Alcalase hydrolysates from casein efficiently 356 increased the intracellular antioxidant enzymes superoxide dismutase (SOD) and catalase 357 (CAT) in H₂O₂ treated HepG2 cells (Xie, Wang, Ao, & Li, 2013). Casein phosphopeptides 358 359 (CPP), obtained by SGID, produced an increase in GSH and CAT activity in H₂O₂ stimulated Caco-2 cells (García-Nebot, Cilla, Alegría, & Barberá, 2011). Prolyve and Alcalase, are food-360 grade proteolytic enzyme preparation obtained from Bacillus licheniformis and both have 361 subtilisin activity. However, only Alcalase possesses glutamyl endopeptidase activity and is 362 consequently able to yield higher extents of hydrolysis than Prolyve (Spellman, Kenny, 363 O'Cuinn, & Fitzgerald, 2005). Thus, the generation of peptides with different proteinases 364 produces distinctive peptide profiles and therefore this may explain the differing results. 365 366

367 3.4. Genoprotective effect of casein hydrolysates

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The ability of the samples to protect against oxidant-induced DNA damage was determined by the comet assay also called single cell gel electrophoresis. This method measures deoxyribonucleic acid (DNA) strand breaks in the cells. The oxidants used, *t*-BOOH (Fig.1a) and H₂O₂ (Fig. 1b), significantly increased the percentage of DNA damage (% DNA tail). None of the samples decreased the DNA damage induced by t-BOOH (Fig.1a). The Prolyve hydrolysate protected against the genotoxic effects of H₂O₂ (p < 0.05).

The rest of the hydrolysates and NaCN were not significantly different from the mean value obtained for H_2O_2 treatment (Fig.1b).

377	This result may be due to the different mechanisms of action of the two oxidants.
378	H_2O_2 induced cell oxidation is produced by the release of hydroxyl radicals (OH·) and it is an
379	iron dependent reaction, whereas t-BOOH produces lipid peroxidation and it is not iron
380	dependent. Previous studies have shown that casein hydrolysates had no effect on DNA
381	damage induced by H_2O_2 in Caco-2 cells (Phelan et al., 2009b). However, several
382	investigations have shown the genoprotective results of food-derived hydrolysates. The
383	enzymatic extracts from a brown seaweed Ecklonia cava showed potent DNA protection in
384	rat lymphocytes using the comet assay (Heo, Park, Park, Kim, & Jeon, 2005). Another study
385	using fish gelatine hydrolysates demonstrated that DNA damage was decreased in a dose-
386	response manner in H ₂ O ₂ challenged U937 cells (Karnjanapratum, O'Callaghan, Benjakul, &
387	O'Brien, 2016). A fractionated protein hydrolysate from brewers' spent grain (BSG) with a
388	molecular mass <5 kDa was reported to decrease the DNA damage in U937 cells treated with
389	H_2O_2 (McCarthy et al., 2013). The authors stated that the genoprotective effect of
390	unfractionated BSG samples was lower than their correspondent fractionated samples. This
391	suggests that further fractionation of the present hydrolysate samples may be of interest to
392	assess specific peptide effects on cellular DNA damage.

393

- 394 3.5. In vitro antioxidant assays
- 395

The chemical antioxidant activity of intact NaCN, the TGase treated NaCN and non-TGase-treated NaCN hydrolysates was determined using four different assays. ORAC, DPPH and ABTS are radical scavenging assays whereas FRAP is based on the ability of the test compound to reduce ferric ions. The results obtained are shown in Fig. 2. The three

400	hydrolysate preparations (Prolyve, TGase/Prolyve and Prolyve/TGase) had significantly
401	higher ($p < 0.05$) ORAC activity than unhydrolysed NaCN. The highest mean ORAC value
402	$(887.1 \pm 52.6 \ \mu mol \ TE \ g^{-1})$ was found in Prolyve/TGase although no significant differences
403	were found between the three hydrolysates. A similar trend was observed using the FRAP
404	assay. The NaCN hydrolysates showed significantly higher FRAP values (23.02, 24.56 and
405	22.95 µM for Prolyve, TGase/Prolyve and Prolyve/TGase, respectively) than untreated casein
406	(6.29 μ M). But again, no differences were found whether the samples were TGase treated or
407	not. FRAP is an antioxidant assay that measures the ability of the hydrolysates to reduce Fe^{3+}
408	to Fe^{2+} . The ion is captured and the chain reaction of the oxidation process does not occur.
409	FRAP values are relatively high in whey proteins. Bagheri, Madadlou, Yarmand, and
410	Mousavi (2014) reported, using the ferric reducing power assay, that cross-linked whey
411	hydrolysates had higher antioxidant activity than those non-cross-linked or intact whey
412	protein. According to Bagheri et al. (2014) cross-linking was responsible for creating peptide
413	structures with the ability of neutralise the ion radicals. However, the results herein
414	demonstrate that the TGase treatment did not affect the FRAP values obtained for TGase-
415	treated NaCN hydrolysates. The FRAP and H_2O_2 -induced DNA damage (Comet assay)
416	assays are both related to an iron-dependent mechanism. Although it was previously shown
417	that the Prolyve hydrolysate had a significant effect on the protection of H_2O_2 -induced DNA
418	damage, the FRAP results showed no difference between non-TGase-treated (Prolyve) and
419	TGase-treated hydrolysates (TGase/Prolyve and Prolyve/TGase). This may be caused by the
420	participation of different components or behaviors of the cells such as enzymatic complexes
421	or cell uptake that could influence the antioxidant response and show diverse results than the
422	in vitro chemical assays (López-Alarcón & Denicola, 2013). DPPH and ABTS inhibition
423	showed no significant differences ($p > 0.05$) between the hydrolysates and intact casein.
424	DPPH is a proton-radical scavenging assay. Some studies have previously shown the

425 potential of casein hydrolysates to scavenge DPPH radical ions (Suetsuna, Ukeda, & Ochi, 2000). However, the hydrolysates generated in the present study were not found to possess 426 DPPH scavenging activity in comparison with Trolox and ascorbic acid. Similar results for 427 428 NaCN hydrolysates were reported by Lahart et al. (2011). The DPPH assay uses methanol or ethanol as solvent. Previous studies determined that the hydrolysate samples generated herein 429 had a hydrophilic profile (data not shown), which may be the reason for the negative results. 430 A method based on an aqueous system, ABTS⁺ assay, was then performed. However, the 431 results showed that the activity of the hydrolysates against ABTS⁺ radical ranged between 6.4 432 and 8.4 % inhibition and none of the hydrolysates showed significant differences with NaCN. 433 A study on the antioxidant activity of different amino acids assessed by the ABTS+ assay, 434 reported that Cys was the most active amino acid followed by Trp, Tyr and His (Aliaga & 435 Lissi, 2000). These specific amino acids are not present in large amounts in caseins. 436 Therefore, based on the results and the amino acid composition of casein it would appear that 437 the DPPH and ABTS⁺ assays may not be adequate methods to quantify the antioxidant 438 potential of casein hydrolysates. It is been reported that the combination of 439 glycosylation/glycation and TGase may increase the antioxidant properties of fish gelatin 440 hydrolysates (Hong et al., 2014). The TGase/treated glycopeptides were shown to inhibit lipid 441 oxidation of linoleic acid and to increase the cellular antioxidant activity in HepG2 cells 442 using the DCFH-DA method. However, the study was performed using guinea pig TGase and 443 444 Alcalase. The authors also explained that the glycosylation/glycation process could be an enhancer of the antioxidant properties of the hydrolysates. 445 The results shown herein demonstrate that casein hydrolysates may be a good source 446 of antioxidant hydrolysates. However, treatment with TGase prior to or post hydrolysis with 447 Prolyve does not seem to exert any significantly difference on the antioxidant activity of the 448

449 hydrolysates. This was consistent across the in vitro antioxidant assays employed herein.

4. Conclusion

453	The findings from the present study show that casein derived peptides may exert
454	specific antioxidant and anti-inflammatory effects. The hydrolysates possessed a higher
455	ORAC and FRAP antioxidant activity in comparison with the unhydrolysed NaCN but the
456	results suggest that the addition of TGase prior to or followed hydrolysis does not change the
457	antioxidant activity of the hydrolysates. The non-TGase-treated hydrolysate sample Prolyve,
458	demonstrated genoprotective activity against H_2O_2 induced DNA damage. On the other hand,
459	the hydrolysates generated with the addition of TGase prior to and after hydrolysis showed a
460	significant decrease in the release of IL-6 cytokine at low concentrations, corresponding to an
461	anti-inflammatory activity. The fractionation and isolation of peptides from these bioactive
462	hydrolysates is the next step to obtain potent immunomodulatory or antioxidant peptides and
463	to incorporate them into functional foods.
464	
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466	
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470	
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Figure legends

Fig. 1. DNA damage (%) in the U937 cell line after incubation with (a) t-BOOH (400 μ M) and (b) hydrogen peroxide (50 μ M). In each graph, different letters denote significant differences between samples at *p* < 0.05 (n=4). Control - : non-treated cells.

Fig. 2. In vitro antioxidant activities of sodium caseinate (NaCN) hydrolysates against: (a) oxygen radical absorbance capacity assay (ORAC, in µmol TROLOX eq g⁻¹); (b) ferric reducing antioxidant power (FRAP) activity; (c) 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition; (d) 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS+) inhibition. Different letters denote significant differences between samples within each graph at p < 0.05 (n=3).

Effect of sodium casein (NaCN), NaCN non-cross-linked hydrolysate (Prolyve) and NaCN transglutaminase (TGase) cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase) on cell viability in the Jurkat T cell line.^a

Concentration	NaCN	Prolyve	TGase/Prolyve	Prolyve/TGase
(%, v/v)				
Control	100.00 ± 0.00	100.00 ± 0.00	100.00±0.00	100.00 ± 0.00
0.025	94.36±6.42	91.40±2.06	95.24±5.56	86.14±3.53
0.05	88.18±5.65	89.61±1.27	87.29±3.49	85.22±2.70*
0.1	$80.60 \pm 8.65*$	90.34±1.17	83.78±2.28	75.69±3.51*
0.5	$68.47 \pm 4.55*$	77.59±5.40*	68.22±4.02*	76.10±4.16*
1.0	72.35±5.56*	$82.80{\pm}6.65$	80.80 ± 7.82	81.07±4.34
2.0	59.79±0.55*	59.38±7.50*	47.46±8.71*	54.16±8.73*
5.0	18.85±5.53*	25.70±16.18*	8.56±0.32*	10.55±1.67*

^a Cells were exposed to increasing concentrations (0.25–50 mg mL⁻¹) of samples for 24 h and cell viability was determined by the MTT assay. Data represent the mean \pm SE of at least three independent experiments expressed as a percentage relative to untreated cells. An asterisk indicates statistically significant difference (p < 0.05) in cell viability between control (untreated) and treated cells.

Table 2

Effect of sodium casein (NaCN), NaCN non-cross-linked hydrolysate (Prolyve) and NaCN transglutaminase (TGase) cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase) on cell viability in the U937 cell line.^a

Concentration	NaCN	Prolyve	TGase/Prolyve	Prolyve/TGase
(%, v/v)				
Control	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
0.025	99.46 ± 2.02	$108.88 \pm 1.74*$	102.04 ± 2.04	95.19±2.81
0.05	102.21 ± 4.00	112.35±3.47	107.29±7.43	95.47±9.26
0.1	93.52±3.64	116.15 ± 5.50	103.78±3.03	105.37±6.25
0.5	100.70 ± 4.46	117.87±2.35	106.96±8.12	106.22±4.26
1.0	91.99±1.53	81.93±4.16	98.39±8.39	79.97±5.44*
2.0	70.60±3.37*	15.34±3.90*	51.98±12.01*	25.02±7.78*
5.0	28.65±2.45*	12.69±1.46*	14.09±1.43*	13.94±1.48*

^a Cells were exposed to increasing concentrations $(0.25-50 \text{ mg mL}^{-1})$ of samples for 24 h and cell viability was determined by the MTT assay. Data represent the mean ± SE of at least three independent experiments expressed as a percentage relative to untreated cells. An asterisk indicates statistically significant difference (p < 0.05) in cell viability between control (untreated) and treated cells.

EC50 values of sodium casein (NaCN), NaCN non-cross-linked hydrolysate (Prolyve) and

NaCN transglutaminase (TGase) cross-linked hydrolysates (TGase/Prolyve and

Prolyve/TGase).^a

Sample	$EC_{50} (mg mL^{-1})$)
	Jurkat T	U937
NaCN	18.65 ^{ac}	31.55 ^a
Prolyve	30.55 ^b	13.96 ^b
TGase/Prolyve	16.02 ^{ac}	21.58 ^c
Prolyve/TGase	22.92 ^{abc}	14.94 ^{bc}

^a Values are mean of at least three independent experiments. Different superscript letters denote significant difference (p < 0.05) for each cell line. EC₅₀ values represent the concentration of sample that inhibits 50% of cell proliferation.

CER C

Effect of sodium casein (NaCN), NaCN non-cross-linked hydrolysate (Prolyve) and NaCN transglutaminase (TGase) cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase) on IL-2 and IL-6 cytokine production in Concanavalin (ConA) stimulated Jurkat T cells. ^a

Sample	ampleCytokine production (% of control)			ntrol)	ol)			
	IL-2				IL-6			
	0.5 mg mL^{-1}		1 mg L ⁻¹		0.5 mg mI	-1	1 mg mL ⁻	1
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	100.00^{a}	0.00	100.00 ^a	0.00	100.00 ^a	0.00	100.00 ^a	0.00
NaCN	107.41 ^a	9.41	79.00 ^b	3.35	41.85 ^b	2.82	30.21 ^b	6.90
Prolyve	98.49 ^a	2.72	93.18 ^a	4.71	88.78 ^{ac}	2.05	80.79 ^c	2.03
TGase/Prolyve	106.17 ^a	3.14	98.38 ^a	5.21	85.45 ^c	2.85	78.12 ^c	1.94
Prolyve/TGase	101.91 ^a	2.98	96.94 ^a	3.44	83.91 ^c	3.71	79.80 ^c	4.81

^a Values are mean \pm SE of at least 3 independent experiments, expressed as a percentage relative to the control (non-treated ConA stimulated cells). Different superscript letters denote significant differences (p < 0.05) in cytokine production between samples.

Glutathione (GSH) content of U937 cells exposed to sodium casein (NaCN), NaCN noncross-linked hydrolysate (Prolyve) and cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase). ^a

Sample	GSH content
Control	100.0±0.0
NaCN	72.1±14.7
Prolyve	111.1±18.6
TGase/Prolyve	134.1±21.8
Prolyve/TGase	130.8±21.7

^a Data are the mean of three independent experiments \pm SE; none of the results was statistically

significant compared with the untreated cells.



