

**UCC Library and UCC researchers have made this item openly available.
Please [let us know](#) how this has helped you. Thanks!**

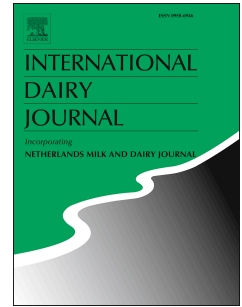
Title	In vitro antioxidant and immunomodulatory activity of transglutaminase-treated sodium caseinate hydrolysates
Author(s)	Cermeño, Maria; FitzGerald, Richard J.; O'Brien, Nora M.
Publication date	2016-08-31
Original citation	Cermeño, M., FitzGerald, R.J. and O'Brien, N.M. (2016) 'In vitro antioxidant and immunomodulatory activity of transglutaminase-treated sodium caseinate hydrolysates', International Dairy Journal, 63, pp. 107-114. doi: 10.1016/j.idairyj.2016.08.007
Type of publication	Article (peer-reviewed)
Rights	© 2016, Elsevier B.V. This manuscript version is made available under the CC-BY-NC-ND 4.0 license. http://creativecommons.org/licenses/by-nc-nd/4.0/
Embargo information	Access to this article is restricted until 12 months after publication by request of the publisher.
Embargo lift date	2017-08-31
Item downloaded from	http://hdl.handle.net/10468/3104

Downloaded on 2021-06-22T14:48:29Z

Accepted Manuscript

In vitro antioxidant and immunomodulatory activity of transglutaminase-treated sodium caseinate hydrolysates

Maria Cermeño, Richard J. FitzGerald, Nora M. O'Brien



PII: S0958-6946(16)30253-9

DOI: [10.1016/j.idairyj.2016.08.007](https://doi.org/10.1016/j.idairyj.2016.08.007)

Reference: INDA 4067

To appear in: *International Dairy Journal*

Received Date: 12 February 2016

Revised Date: 4 July 2016

Accepted Date: 19 August 2016

Please cite this article as: Cermeño, M., FitzGerald, R.J., O'Brien, N.M., In vitro antioxidant and immunomodulatory activity of transglutaminase-treated sodium caseinate hydrolysates, *International Dairy Journal* (2016), doi: 10.1016/j.idairyj.2016.08.007.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

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

3

4

5

6

7

8 Maria Cermeño^{a,b}, Richard J. FitzGerald^a, Nora M. O'Brien^{b*}

9

10

11

12

13

14 ^a *Department of Life Sciences, University of Limerick, Limerick, Ireland*

15 ^b *School of Food and Nutritional Sciences, University College Cork, Cork, Ireland*

16

17

18

19

20

21

22 *Corresponding author. Tel.: +353 21 4902884

23 *E-mail address:* nob@ucc.ie (N. M. O'Brien)

24

25

26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

ABSTRACT

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

51 1. Introduction

52

53 Approximately 30% of occidental population deaths are due to diseases related to
54 cardiovascular problems (WHO, 2011). The continuous exposure to chemicals, unhealthy
55 diets and sedentary life-style may be contributing factors for premature ageing and illness.
56 Oxidative stress is an imbalance between the production of free radicals or reactive oxygen
57 species (ROS) and the neutralisation of these by antioxidant compounds (Lobo, Patil, Phatak,
58 & Chandra, 2010). The excess of ROS produced as a result of oxidative stress is involved in
59 the pathogenesis of neurodegenerative, cardiovascular and inflammatory diseases. For
60 instance, atherosclerotic cardiovascular disease is characterised by the oxidation of low-
61 density lipoproteins (LDL) which induce the adhesion and influx of monocytes and lead to
62 cytokine production, a pro-inflammatory response (Singh, Devaraj, & Jialal, 2005). Some
63 multifactorial diseases such as atherosclerosis or Parkinson's disease are the result of
64 combined inflammatory and oxidative processes (Chen, Lü, Yao, & Chen, 2016). For this
65 reason, there is an increasing interest in studies on the anti-inflammatory and antioxidant
66 potential of bioactive dietary ingredients.

67 Bioactive peptides (BAPs) are natural protein fragments obtained from food proteins
68 such as dairy, eggs, fish, meat or vegetables. These peptides can be released from proteins by
69 bacterial fermentation, digestion or enzymatic hydrolysis and they may possess potent
70 bioactivities (Korhonen & Pihlanto, 2006; Nongonierma, O'Keeffe, & FitzGerald, 2016).
71 Antioxidant BAPs may inhibit the action of free radicals, reducing oxidation events and
72 thereby contribute to the prevention of inflammatory responses. Antioxidant and anti-
73 inflammatory bioactivities are directly related (Pashkow, 2011). Caseins from bovine milk
74 contain a large number of bioactive peptides encrypted into the parent protein (Hernández-
75 Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014; Nongonierma &

76 FitzGerald, 2015; Nongonierma et al., 2016; Phelan, Aherne, FitzGerald, & O'Brien, 2009a;
77 Power, Jakeman, & FitzGerald, 2012; Wada & Lönnerdal, 2014). The composition, structure,
78 hydrophobicity, position of amino acid residue and molecular mass are factors directly
79 related with the activity of BAPs (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara,
80 1998). The amino acid composition of casein, which is rich in Pro residues, makes it a
81 potential source of bioactive peptides for the production of biofunctional foods (Pihlanto,
82 2006).

83 Enzymatic hydrolysis of casein proteins has resulted in the generation of BAPs with
84 demonstrated immunomodulatory and antioxidant activities. Two casein hydrolysates,
85 deriving from digestion with *Lb. helveticus* MIMLh5 and *Lb. acidophilus* ATCC 4356
86 proteinases, demonstrated anti-inflammatory activity by decreasing NF- κ B activity in
87 recombinant Caco-2 cells (Stuknyte, De Noni, Guglielmetti, Minuzzo, & Mora, 2011). A
88 recent study demonstrated that a <5 kDa NaCN hydrolysate was able to reduce IL-8, a pro-
89 inflammatory cytokine, in tumour necrosis factor-alpha (TNF- α) treated Caco-2 cells, and
90 similar results were observed ex vivo in porcine colonic tissue (Mukhopadhyaya et al., 2015).
91 Similarly, a peptide obtained from β -CN (f 94-98), QEPVL, and its derivative, QEPV,
92 showed the capacity to regulate the inflammatory process not only in vitro but also in vivo in
93 Balb/c mice (Jiehui et al., 2014). Studies using Balb/c mice reported that yak casein
94 hydrolysates possessed radical scavenging activities against 2,2-Diphenyl-1-picrylhydrazyl
95 (DPPH), superoxide and hydrogen peroxide, and also decreased the production of nitric oxide
96 (NO) and the pro-inflammatory IL-6 and IL-1 β cytokines (Mao, Cheng, Wang, & Wu, 2011).
97 The antioxidant properties of casein hydrolysates have been widely reviewed (Pihlanto, 2006;
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
102 improve the physicochemical and organoleptic properties of dairy products. The addition of
103 TGase improved the emulsifying and foaming properties of NaCN (Flanagan & FitzGerald,
104 2003). The application of TGase in yoghurt and cheese is well established leading to
105 improved product quality (Özer, Hayaloglu, Yaman, Gürsoy, & Şener, 2013; Romeih, Abdel-
106 Hamid, & Awad, 2014). However, little is known about the effect of TGase on the bioactivity
107 of peptides. A recent study by Hong, Gottardi, Ndagijimana, and Betti (2014) found that
108 glycopeptides from fish, obtained by glycosylation and proteolytic hydrolysis with Alcalase,
109 improved their cellular antioxidant activity in HepG2 cells and their lipid oxidation inhibition
110 activity with the addition of TGase. Additionally, gluten hydrolysates glycosylated with
111 TGase, have been reported to improve their in vitro antioxidant activity (Gottardi, Hong,
112 Ndagijimana, & Betti, 2014). Preliminary work in our laboratory has shown that samples
113 treated with TGase prior to hydrolysis had an anti-inflammatory activity in LPS induced
114 Jurkat T cells; however, no antioxidant activity was detected (O'Sullivan, Lahart,
115 O'Callaghan, O'Brien, & FitzGerald, 2013).

116 The aim of the present study was to assess the effect of enzymatic hydrolysis and its
117 combination with TGase cross-linking treatment on the immunomodulatory and antioxidant
118 activity of NaCN hydrolysates.

119

120 **2. Materials and methods**

121

122 *2.1. Materials*

123

124 Sodium caseinate (NaCN; 87.57%, (w/w, protein) was provided by Arrabawn Co-op
125 Society Ltd., Tipperary, Ireland. Calcium independent TGase from *Streptovercillium* spp.

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.

134

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™ at 50 °C
140 and pH 7 using a pH stat (Titrand 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™ using the same conditions as
147 outlined above. All the hydrolysates generated were 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® (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).

151

152 2.3. *Cell culture*

153

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% CO₂ 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 µL) and MTT reagent 1 (5 µL) was added and cells were incubated for 4 h at 37 °C.
166 Then, MTT reagent 2 (100 µL) 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 (EC₅₀) 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 µg L⁻¹) and were incubated with test samples at 0.5

176 and 1 mg mL^{-1} for 24 h at $37 \text{ }^{\circ}\text{C}$. Production of the cytokines IL-6 and IL-2 was determined
177 using ELISA kits. Absorbance was read at 450 nm using a microplate reader. Experiments
178 were performed in triplicate ($n = 3$) and data were expressed as a percentage of the stimulated
179 cell control.

180

181 2.6. *Antioxidant activity*

182

183 2.6.1. *Intracellular reduced glutathione (GSH)*

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

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 $37 \text{ }^{\circ}\text{C}$. After incubation, cells were treated
198 with $50 \text{ } \mu\text{M}$ H_2O_2 or $400 \text{ } \mu\text{M}$ 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 °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 µg 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
210 by Zulueta, Esteve, and Frígola (2009) using a Synergy™ HT plate reader (BioTek
211 Instruments Limited, Bedfordshire, UK). An aliquot (50 µL) of test sample (0.1 mg mL⁻¹),
212 standard or phosphate buffer (75 mM) and 50 µL of fluorescein (0.78 µM) were added into a
213 microtitre plate incubated at 37 °C. The reaction was started with the addition of 25 µL of
214 2,2'-azobis-2-methyl-propanimidamide (AAPH) to each well. Fluorescence readings were
215 recorded every 5 min for 120 min at excitation and emission wavelengths of 485 and 520 nm,
216 respectively. The ORAC values, expressed as µmoles trolox equivalents (TE) per mg freeze
217 dried sample, were calculated using trolox as a standard. Experiments were performed in
218 triplicate (n = 3).

219

220 2.6.4. *Ferric reducing antioxidant power activity*

221 The ferric reducing antioxidant power (FRAP) value of hydrolysate samples was
222 determined using the method described by Benzie and Strain (1999) with some
223 modifications. Briefly, 2 mL of freshly prepared FRAP reagent [150 µL; 0.3 M acetate buffer
224 (pH 3.6), 0.01 M 2,4,6-tripyridyl-s-triazine (TPTZ), 0.02 M FeCl₃·6H₂O 10:1:1] heated to
225 37 °C was added into a cuvette and the absorbance was read at 590 nm. Test sample

226 (100 μL), FeSO_4 (standard) and MeOH (blank) was then added and the absorbance (590 nm)
227 was read after 30 min incubation at 37 $^\circ\text{C}$. The experiment was performed in triplicate ($n =$
228 3) and the FRAP values (μM) were calculated from the standard curve.

229

230 2.6.5. 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonate) radical scavenging assay

231 The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate) (ABTS+) radical scavenging
232 activities were measured using the previously described method by Re et al. (1999). The
233 ABTS \bullet + solution was prepared by mixing 7 mM ABTS with 2.45 mM potassium persulphate
234 for 16 h to generate the radicals. The radical solution was then diluted to an absorbance of
235 0.70 ± 0.02 at 734 nm. Test samples (10 μL) at a final concentration 1 mg mL^{-1} were added in
236 a 96 well plate with the radical solution (200 μL) and kept in the dark at room temperature
237 for 5 min. Absorbance was then measured at 734 nm. Known concentrations of trolox
238 solutions were used to plot a standard curve and ABTS+ values were calculated. All samples
239 were analysed in triplicate ($n=3$) and the final inhibitory activity was expressed as % ABTS
240 inhibition using the following equation:

$$241 \quad \text{ABTS inhibitory activity (\%)} = [(\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{blank}}] \times 100$$

242

243 2.6.6. DPPH radical scavenging assay

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

251 DPPH inhibition (%) = $[(\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}) / \text{Abs}_{\text{blank}}] \times 100$

252

253 **2.7. Statistical analysis**

254

255 All data were determined as the mean and standard error values of at least three
256 independent experiments. Data were analysed by one-way analysis of variance (ANOVA)
257 followed by Dunnett's test (or Tukey's multiple comparison test, where appropriate), using
258 Graph-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 experiments. The MTT assay measures cellular mitochondrial activity by assessing the
267 activity of mitochondrial reductase. Cells were incubated with increasing concentrations of
268 NaCN and its hydrolysates (0–5%, v/v, equivalent to 0–50 mg mL⁻¹). The EC₅₀ values were
269 calculated and represent the concentration required that inhibits cell viability by 50%. Based
270 on the EC₅₀ values obtained, the hydrolysates seemed to have similar cytotoxic effects on
271 Jurkat T and U937 cells (Table 3). McCarthy et al. (2013) reported the cytotoxic effect of
272 brewers' spent grain (BSG) hydrolysates on U937 cells was higher than on Jurkat T cells. In
273 the present study, samples at 0.5% (v/v) showed significant ($p < 0.05$) inhibition of the
274 viability of Jurkat T cells compared with control (non-treated cells) supporting the previous
275 results 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%
277 compared with untreated cells (100%). All the hydrolysates studied herein induced a
278 significant ($p < 0.05$) cytotoxic activity at concentrations of 0.5% (v/v) in Jurkat T cells. In a
279 study by Phelan, Aherne-Bruce, O'Sullivan, FitzGerald, and O'Brien (2009b), NaCN
280 hydrolysates prepared using different food-grade enzyme preparations were cytotoxic to
281 Jurkat T cells at a concentration of 0.5% (v/v). The addition of $100 \mu\text{g mL}^{-1}$ of a
282 glycomacropeptide from bovine milk was reported to significantly inhibit the viability of
283 U937 cells (Li & Mine, 2004). In contrast, in our study concentrations of the hydrolysates up
284 to 5 mg mL^{-1} (corresponding to 0.5%, v/v), showed no inhibition in the viability of U937
285 cells. The conformation, degree of hydrolysis and the source of the proteolytic enzyme used
286 to generate the hydrolysates are key factors that may affect the cytotoxicity of hydrolysates
287 (Lahart et al., 2011; Zou, He, Li, Tang, & Xia, 2016). TGase treated hydrolysates showed
288 similar results in Jurkat T cells to those reported by O'Sullivan et al. (2013). Sample
289 concentrations that showed a cell viability $< 75\%$ of control were considered toxic.
290 Therefore, non-toxic test sample concentrations of 0.5 and 1 mg mL^{-1} were used for
291 subsequent immunomodulatory and antioxidant activities in both cell lines.

292

293 3.2. *Immunomodulatory effects of NaCN hydrolysates*

294

295 The anti-inflammatory activity of intact NaCN, the NaCN hydrolysate (Prolyve) and
296 the cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase) was screening by
297 measuring their potential to suppress the production of IL-2 and IL-6 in ConA stimulated
298 Jurkat T cells. ConA is a lectin mitogen known for its ability to stimulate the T-cell receptor
299 and the subsequent activation of signalling pathways involving nuclear factor of activated T-
300 cells (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 ($p <$
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
327 ConA-stimulated Jurkat T cells incubated with NaCN cross-linked with TGase pre-
328 hydrolysis. The extent of hydrolysis reached and the enzymatic preparation used to generate
329 the hydrolysates are mainly responsible for the final sequences of peptides within the
330 hydrolysates and could induce different cell reactions. It is interesting to note a study where
331 the substitution of proline in short peptides had a negative effect on their immunomodulatory
332 activity, but the substitution of proline with proline analogues did not have an impact on the
333 final bioactivity. The study reported on an immunomodulatory peptide from β -casein (191–
334 209) (LLYQEPVLGPVRGPFPIIV) which was synthesised with modifications around Pro
335 residues. In particular, substitution of the last proline (P206) with D-Pro produced an
336 inhibition in the in vitro immunosuppressory effects in α -CD3 and α -CD28 stimulated murine
337 spleen cells (Bonomi et al., 2011). Hence, the structure and sequence of peptides is a crucial
338 factor which directly affects their anti-inflammatory activity.

339

340 3.3. Cellular antioxidant assays

341

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

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

366

367 3.4. Genoprotective effect of casein hydrolysates

368

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

375 The rest of the hydrolysates and NaCN were not significantly different from the mean value
376 obtained for H₂O₂ treatment (Fig.1b).

377 This result may be due to the different mechanisms of action of the two oxidants.
378 H₂O₂ 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 H₂O₂ 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 H₂O₂ 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 H₂O₂ (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

396 The chemical antioxidant activity of intact NaCN, the TGase treated NaCN and non-
397 TGase-treated NaCN hydrolysates was determined using four different assays. ORAC, DPPH
398 and ABTS are radical scavenging assays whereas FRAP is based on the ability of the test
399 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 ($887.1 \pm 52.6 \mu\text{mol TE g}^{-1}$) 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 $22.95 \mu\text{M}$ for Prolyve, TGase/Prolyve and Prolyve/TGase, respectively) than untreated casein
406 ($6.29 \mu\text{M}$). 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,
426 2000). However, the hydrolysates generated in the present study were not found to possess
427 DPPH scavenging activity in comparison with Trolox and ascorbic acid. Similar results for
428 NaCN hydrolysates were reported by Lahart et al. (2011). The DPPH assay uses methanol or
429 ethanol as solvent. Previous studies determined that the hydrolysate samples generated herein
430 had a hydrophilic profile (data not shown), which may be the reason for the negative results.
431 A method based on an aqueous system, ABTS⁺ assay, was then performed. However, the
432 results showed that the activity of the hydrolysates against ABTS⁺ radical ranged between 6.4
433 and 8.4 % inhibition and none of the hydrolysates showed significant differences with NaCN.
434 A study on the antioxidant activity of different amino acids assessed by the ABTS⁺ assay,
435 reported that Cys was the most active amino acid followed by Trp, Tyr and His (Aliaga &
436 Lissi, 2000). These specific amino acids are not present in large amounts in caseins.
437 Therefore, based on the results and the amino acid composition of casein it would appear that
438 the DPPH and ABTS⁺ assays may not be adequate methods to quantify the antioxidant
439 potential of casein hydrolysates. It is been reported that the combination of
440 glycosylation/glycation and TGase may increase the antioxidant properties of fish gelatin
441 hydrolysates (Hong et al., 2014). The TGase/treated glycopeptides were shown to inhibit lipid
442 oxidation of linoleic acid and to increase the cellular antioxidant activity in HepG2 cells
443 using the DCFH-DA method. However, the study was performed using guinea pig TGase and
444 Alcalase. The authors also explained that the glycosylation/glycation process could be an
445 enhancer of the antioxidant properties of the hydrolysates.

446 The results shown herein demonstrate that casein hydrolysates may be a good source
447 of antioxidant hydrolysates. However, treatment with TGase prior to or post hydrolysis with
448 Prolyve does not seem to exert any significantly difference on the antioxidant activity of the
449 hydrolysates. This was consistent across the in vitro antioxidant assays employed herein.

450

451 **4. Conclusion**

452

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 H₂O₂ 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

465 **Acknowledgements**

466

467 Funding for this research was provided under the National Development Plan 2007-
468 2013, through the Food Institutional Research Measure (FIRM), administered by the
469 Department of Agriculture, Food and Marine, Ireland under grant issue 10 RD TMFRC 701.

470

471 **References**

472

- 473 Aliaga, C., & Lissi, E. A. (2000). Reactions of the radical cation derived from 2,2'-
474 azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS^{·+}) with amino acids.
475 Kinetics and mechanism. *Canadian Journal of Chemistry*, 78, 1052–1059.
- 476 Aluko, R. E. (2012). *Functional foods and nutraceuticals* (Vol. 23). New York, NY, USA:
477 Springer.
- 478 Bagheri, L., Madadlou, A., Yarmand, M., & Mousavi, M. E. (2014). Potentially bioactive and
479 caffeine-loaded peptidic sub-micron and nanoscalar particles. *Journal of Functional*
480 *Foods*, 6, 462–469.
- 481 Benzie, I. F., & Strain, J. J. (1999). Ferric reducing/antioxidant power assay: direct measure
482 of total antioxidant activity of biological fluids and modified version for simultaneous
483 measurement of total antioxidant power and ascorbic acid concentration. *Methods in*
484 *Enzymology*, 299, 15–27.
- 485 Bonomi, F., Brandt, R., Favalli, S., Ferranti, P., Fierro, O., Frøkiær, H., et al. (2011).
486 Structural determinants of the immunomodulatory properties of the C-terminal region
487 of bovine β -casein. *International Dairy Journal*, 21, 770–776.
- 488 Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to
489 evaluate antioxidant activity. *LWT - Food Science and Technology*, 28, 25–30.
- 490 Chen, A. Y., Lü, J.-M., Yao, Q., & Chen, C. (2016). Entacapone is an antioxidant more
491 potent than vitamin C and vitamin E for scavenging of hypochlorous acid and
492 peroxynitrite, and the inhibition of oxidative stress-induced cell death. *Medical*
493 *Science Monitor*, 22, 687–696.
- 494 Chen, H.-M., Muramoto, K., Yamauchi, F., Fujimoto, K., & Nokihara, K. (1998).
495 Antioxidative properties of histidine-containing peptides designed from peptide
496 fragments found in the digests of a soybean protein. *Journal of Agricultural and Food*
497 *Chemistry*, 46, 49–53.

- 498 Flanagan, J., & FitzGerald, R. J. (2003). Functional properties of *Bacillus* proteinase
499 hydrolysates of sodium caseinate incubated with transglutaminase pre- and post-
500 hydrolysis. *International Dairy Journal*, *13*, 135–143.
- 501 García-Nebot, M. J., Cilla, A., Alegría, A., & Barberá, R. (2011). Caseinophosphopeptides
502 exert partial and site-specific cytoprotection against H₂O₂-induced oxidative stress in
503 Caco-2 cells. *Food Chemistry*, *129*, 1495–1503.
- 504 Gottardi, D., Hong, P. K., Ndagijimana, M., & Betti, M. (2014). Conjugation of gluten
505 hydrolysates with glucosamine at mild temperatures enhances antioxidant and
506 antimicrobial properties. *LWT - Food Science and Technology*, *57*, 181–187.
- 507 Heo, S.-J., Park, P.-J., Park, E.-J., Kim, S.-K., & Jeon, Y.-J. (2005). Antioxidant activity of
508 enzymatic extracts from a brown seaweed *Ecklonia cava* by electron spin resonance
509 spectrometry and comet assay. *European Food Research and Technology*, *221*, 41–
510 47.
- 511 Hernández-Ledesma, B., García-Nebot, M. J., Fernández-Tomé, S., Amigo, L., & Recio, I.
512 (2014). Dairy protein hydrolysates: Peptides for health benefits. *International Dairy*
513 *Journal*, *38*, 82–100.
- 514 Hong, P. K., Gottardi, D., Ndagijimana, M., & Betti, M. (2014). Glycation and
515 transglutaminase mediated glycosylation of fish gelatin peptides with glucosamine
516 enhance bioactivity. *Food Chemistry*, *142*, 285–293.
- 517 Jiehui, Z., Liuliu, M., Haihong, X., Yang, G., Yingkai, J., Lun, Z., et al. (2014).
518 Immunomodulating effects of casein-derived peptides QEPVL and QEPV on
519 lymphocytes in vitro and in vivo. *Food and Function*, *5*, 2061–2069.
- 520 Karnjanapratum, S., O'Callaghan, Y. C., Benjakul, S., & O'Brien, N. (2016). Antioxidant,
521 immunomodulatory and antiproliferative effects of gelatin hydrolysate from unicorn
522 leatherjacket skin. *Journal of the Science of Food and Agriculture*, *96*, 3220–3226.

- 523 Korhonen, H., & Pihlanto, A. (2006). Bioactive peptides: Production and functionality.
524 *International Dairy Journal*, 16, 945–960.
- 525 Lahart, N., O’Callaghan, Y., Aherne, S. A., O’Sullivan, D., FitzGerald, R. J., & O’Brien, N.
526 M. (2011). Extent of hydrolysis effects on casein hydrolysate bioactivity: Evaluation
527 using the human Jurkat T cell line. *International Dairy Journal*, 21, 777–782.
- 528 Li, E. W. Y., & Mine, Y. (2004). Immunoenhancing effects of bovine glycomacropeptide and
529 its derivatives on the proliferative response and phagocytic activities of human
530 macrophage-like cells, U937. *Journal of Agricultural and Food Chemistry*, 52, 2704–
531 2708.
- 532 Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and
533 functional foods: Impact on human health. *Pharmacognosy Reviews*, 4, 118–126.
- 534 López-Alarcón, C., & Denicola, A. (2013). Evaluating the antioxidant capacity of natural
535 products: A review on chemical and cellular-based assays. *Analytica Chimica Acta*,
536 763, 1–10.
- 537 Mao, X. Y., Cheng, X., Wang, X., & Wu, S. J. (2011). Free-radical-scavenging and anti-
538 inflammatory effect of yak milk casein before and after enzymatic hydrolysis. *Food*
539 *Chemistry*, 126, 484–490.
- 540 Mao, X. Y., Yang, H. Y., Song, J. P., Li, Y. H., & Ren, F. Z. (2007). Effect of yak milk
541 casein hydrolysate on Th1/Th2 cytokines production by murine spleen lymphocytes *in*
542 *vitro*. *Journal of Agricultural and Food Chemistry*, 55, 638–642.
- 543 McCarthy, A. L., O’Callaghan, Y. C., Connolly, A., Piggott, C. O., FitzGerald, R. J., &
544 O’Brien, N. M. (2012). Phenolic extracts of brewers’ spent grain (BSG) as functional
545 ingredients – Assessment of their DNA protective effect against oxidant-induced
546 DNA single strand breaks in U937 cells. *Food Chemistry*, 134, 641–646.

- 547 McCarthy, A. L., O'Callaghan, Y. C., Connolly, A., Piggott, C. O., FitzGerald, R. J., &
548 O'Brien, N. M. (2013). Brewers' spent grain (BSG) protein hydrolysates decrease
549 hydrogen peroxide (H₂O₂)-induced oxidative stress and concanavalin-A (con-A)
550 stimulated IFN- γ production in cell culture. *Food and Function*, 4, 1709–1716.
- 551 Mukhopadhyaya, A., Noronha, N., Bahar, B., Ryan, M. T., Murray, B. A., Kelly, P. M., et al.
552 (2015). The anti-inflammatory potential of a moderately hydrolysed casein and its 5
553 kDa fraction in in vitro and ex vivo models of the gastrointestinal tract. *Food and*
554 *Function*, 6, 612–621.
- 555 Nongonierma, A., & FitzGerald, R. (2015). Bioactive properties of milk proteins in humans:
556 A review. *Peptides*, 73, 20–34.
- 557 Nongonierma, A., O'Keeffe, M., & FitzGerald, R. (2016). Milk protein hydrolysates and
558 bioactive peptides. In P. L. H. McSweeney & J. A. O'Mahony (Eds.), *Advanced dairy*
559 *chemistry: Vol. 1B: Proteins: Applied aspects* (pp. 417–482). New York, NY, USA:
560 Springer New York.
- 561 O'Sullivan, D., Lahart, N., O'Callaghan, Y., O'Brien, N. M., & FitzGerald, R. J. (2013).
562 Characterisation of the physicochemical, residual antigenicity and cell activity
563 properties of transglutaminase cross-linked sodium caseinate hydrolysates.
564 *International Dairy Journal*, 33, 49–54.
- 565 O'Sullivan, S. M., O'Callaghan, Y. C., O'Keeffe, M. B., FitzGerald, R. J., & O'Brien, N. M.
566 (2015). Potential immunomodulatory effects of casein-derived bioactive peptides in
567 human T cells. *Proceedings of the Nutrition Society*, 74, e107.
- 568 Özer, B., Hayaloglu, A. A., Yaman, H., Gürsoy, A., & Şener, L. (2013). Simultaneous use of
569 transglutaminase and rennet in white-brined cheese production. *International Dairy*
570 *Journal*, 33, 129–134.

- 571 Pashkow, F. J. (2011). Oxidative stress and inflammation in heart disease: Do antioxidants
572 have a role in treatment and/or prevention? *International Journal of Inflammation*,
573 *2001*, article 514623.
- 574 Phelan, M., Aherne, A., FitzGerald, R. J., & O'Brien, N. M. (2009a). Casein-derived
575 bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory
576 status. *International Dairy Journal*, *19*, 643–654.
- 577 Phelan, M., Aherne-Bruce, S. A., O'Sullivan, D., FitzGerald, R. J., & O'Brien, N. M. (2009b).
578 Potential bioactive effects of casein hydrolysates on human cultured cells.
579 *International Dairy Journal*, *19*, 279–285.
- 580 Pihlanto, A. (2006). Antioxidative peptides derived from milk proteins. *International Dairy*
581 *Journal*, *16*, 1306–1314.
- 582 Power, O., Jakeman, P., & FitzGerald, R. J. (2012). Antioxidative peptides: enzymatic
583 production, in vitro and in vivo antioxidant activity and potential applications of milk-
584 derived antioxidative peptides. *Amino Acids*, *44*, 797–820.
- 585 Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999).
586 Antioxidant activity applying an improved ABTS radical cation decolorization assay.
587 *Free Radical Biology and Medicine*, *26*, 1231–1237.
- 588 Romeih, E. A., Abdel-Hamid, M., & Awad, A. A. (2014). The addition of buttermilk powder
589 and transglutaminase improves textural and organoleptic properties of fat-free buffalo
590 yogurt. *Dairy Science and Technology*, *94*, 297–309.
- 591 Singh, U., Devaraj, S., & Jialal, I. (2005). Vitamin E, oxidative stress and inflammation.
592 *Annual Review of Nutrition*, *25*, 151–174.
- 593 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M.
594 D., et al. (1985). Measurement of protein using bicinchoninic acid. *Analytical*
595 *Biochemistry*, *150*, 76–85.

- 596 Spellman, D., Kenny, P., O'Cuinn, G., & Fitzgerald, R. J. (2005). Aggregation properties of
597 whey protein hydrolysates generated with *Bacillus licheniformis* proteinase activities.
598 *Journal of Agricultural and Food Chemistry*, *53*, 1258–1265.
- 599 Stuknyte, M., De Noni, I., Guglielmetti, S., Minuzzo, M., & Mora, D. (2011). Potential
600 immunomodulatory activity of bovine casein hydrolysates produced after digestion
601 with proteinases of lactic acid bacteria. *International Dairy Journal*, *21*, 763–769.
- 602 Suetsuna, K., Ukeda, H., & Ochi, H. (2000). Isolation and characterization of free radical
603 scavenging activities peptides derived from casein. *Journal of Nutritional*
604 *Biochemistry*, *11*, 128–131.
- 605 Takahashi, K., Murakami, M., Hosaka, K., Kikuchi, H., Oshima, Y., & Kubohara, Y. (2009).
606 Regulation of IL-2 production in Jurkat cells by Dictyostelium-derived factors. *Life*
607 *Sciences*, *85*, 438–443.
- 608 Tanaka, S., Akaishi, E., Hosaka, K., Okamura, S., & Kubohara, Y. (2005). Zinc ions suppress
609 mitogen-activated interleukin-2 production in Jurkat cells. *Biochemical and*
610 *Biophysical Research Communications*, *335*, 162–167.
- 611 Wada, Y., & Lönnnerdal, B. (2014). Bioactive peptides derived from human milk proteins —
612 mechanisms of action. *The Journal of Nutritional Biochemistry*, *25*, 503–514.
- 613 Walsh, D. J., Bernard, H., Murray, B. A., MacDonald, J., Pentzien, A. K., Wright, G. A., et
614 al. (2004). In vitro generation and stability of the lactokinin β -lactoglobulin fragment
615 (142–148). *Journal of Dairy Science*, *87*, 3845–3857.
- 616 WHO. (2011). *Global atlas on cardiovascular disease prevention and control*. Geneva:
617 World Health Organization.
- 618 Xie, N., Wang, C., Ao, J., & Li, B. (2013). Non-gastrointestinal-hydrolysis enhances
619 bioavailability and antioxidant efficacy of casein as compared with its in vitro
620 gastrointestinal digest. *Food Research International*, *51*, 114–122.

- 621 Zhang, Q.-X., Jin, M.-M., Zhang, L., Yu, H.-X., Sun, Z., & Lu, R.-R. (2015). Hydrophobicity
622 of whey protein hydrolysates enhances the protective effect against oxidative damage
623 on PC 12 cells. *Journal of Dairy Research*, 82, 1–7.
- 624 Zou, T.-B., He, T.-P., Li, H.-B., Tang, H.-W., & Xia, E.-Q. (2016). The structure-activity
625 relationship of the antioxidant peptides from natural proteins. *Molecules*, 21, article
626 72.
- 627 Zulueta, A., Esteve, M. J., & Frígola, A. (2009). ORAC and TEAC assays comparison to
628 measure the antioxidant capacity of food products. *Food Chemistry*, 114, 310–316.

Figure legends

Fig. 1. DNA damage (%) in the U937 cell line after incubation with (a) t-BOOH (400 μM) and (b) hydrogen peroxide (50 μM). 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 $\mu\text{mol TROLOX eq g}^{-1}$); (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 $p < 0.05$ ($n=3$).

Table 1

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 (% v/v)	NaCN	Prolyve	TGase/Prolyve	Prolyve/TGase
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±8.65*	90.34±1.17	83.78±2.28	75.69±3.51*
0.5	68.47±4.55*	77.59±5.40*	68.22±4.02*	76.10±4.16*
1.0	72.35±5.56*	82.80±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 ± 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 (%, v/v)	NaCN	Prolyve	TGase/Prolyve	Prolyve/TGase
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±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 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.

Table 3

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.

Table 4

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	Cytokine production (% of control)							
	IL-2				IL-6			
	0.5 mg mL ⁻¹		1 mg L ⁻¹		0.5 mg mL ⁻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.

Table 5

Glutathione (GSH) content of U937 cells exposed to sodium casein (NaCN), NaCN non-cross-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 ± SE; none of the results was statistically significant compared with the untreated cells.

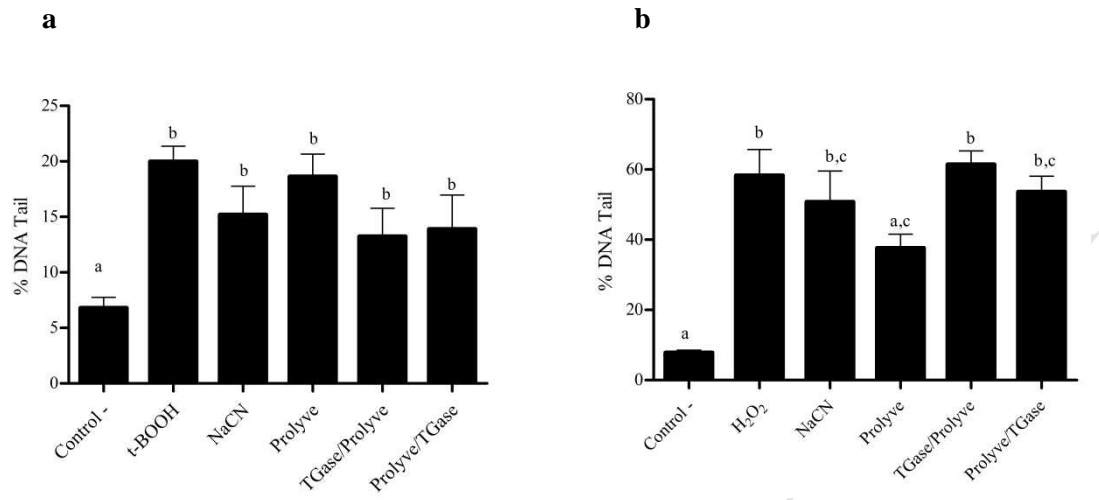


Figure 1.

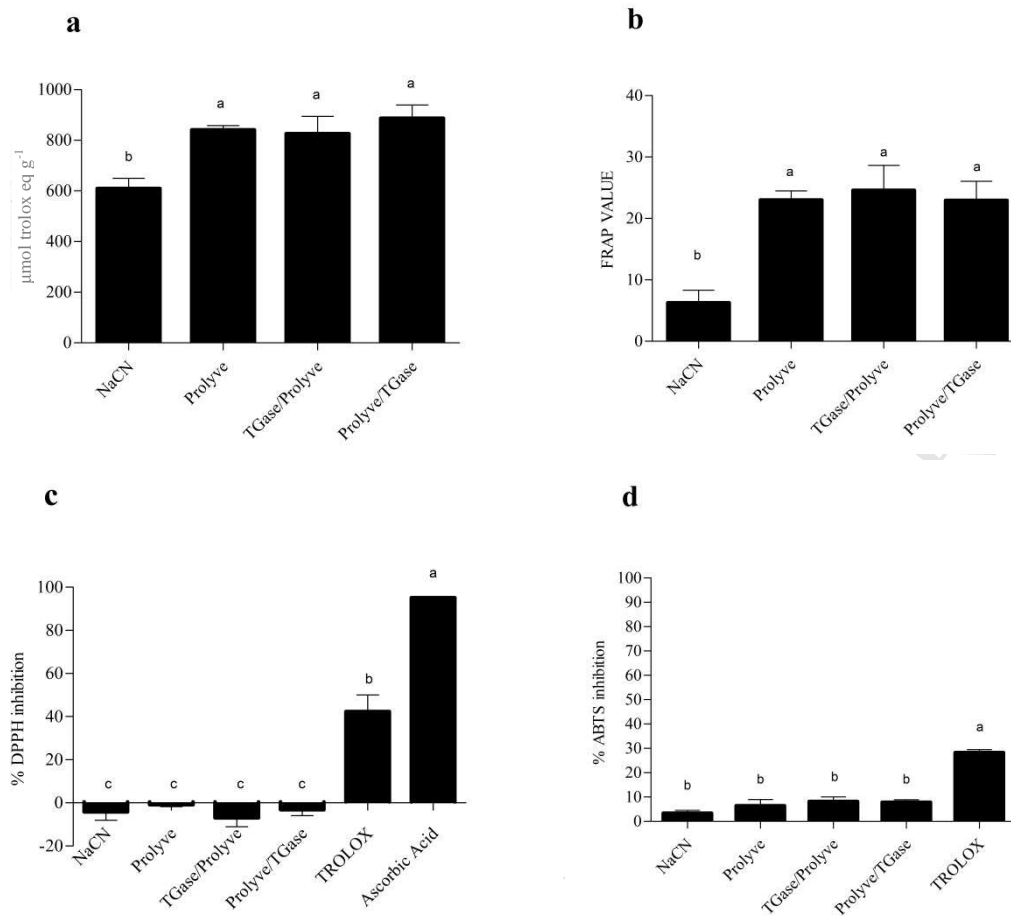


Figure 2.