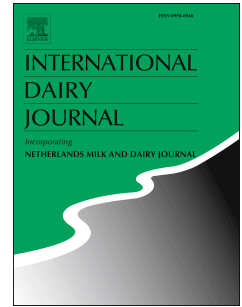


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In vitro antioxidant and immunomodulatory activity of transglutaminase-treated sodium caseinate hydrolysates

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2 **sodium caseinate hydrolysates.**

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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<sup>-1</sup> and the non-TGase treated hydrolysate reduced the production of IL-6 at 1 mg mL<sup>-1</sup> 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.

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## 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 & Lönnnerdal, 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- $\kappa$ 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- $\alpha$ ) 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  $\beta$ -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 $\beta$  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% CO<sub>2</sub> 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  $1 \times 10^5$  cells mL<sup>-1</sup> in growth media were seeded in each well of 96-  
162 well flat-bottom plates. Cells were incubated with hydrolysates (0–50 mg mL<sup>-1</sup>) 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 (EC<sub>50</sub>) values were calculated in triplicate (n = 3) and expressed as mg mL<sup>-1</sup>  
170 using GraphPad Prism 4.

171

172 2.5. *Immunomodulatory activity – cytokine production*

173

174 Jurkat T cells, at a density of  $2 \times 10^5$  cells mL<sup>-1</sup>, were seeded in 96-well plates in the  
175 presence of concanavalin A (ConA, 50 µg L<sup>-1</sup>) and were incubated with test samples at 0.5



176 and  $1 \text{ mg mL}^{-1}$  for 24 h at  $37^\circ\text{C}$ . 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  $37^\circ\text{C}$ . Following incubation, cells were harvested,  
186 sonicated on ice at 13 mA for 30 s, centrifuged ( $14,000 \times g$ , 30 min,  $4^\circ\text{C}$ ) and the supernatant  
187 was collected. An aliquot (100  $\mu\text{L}$ ) 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  $5 \text{ mg mL}^{-1}$  (0.5%, v/v) of test sample  
197 for 24 h in a 6-well plate (final volume 2 mL) at  $37^\circ\text{C}$ . After incubation, cells were treated  
198 with  $50 \mu\text{M H}_2\text{O}_2$  or  $400 \mu\text{M tert-butyl hydroperoxide (t-BOOH)}$  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<sup>-1</sup>). 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<sup>-1</sup>),  
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 FeCl<sub>3</sub>·6H<sub>2</sub>O 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  $\mu\text{L}$ ),  $\text{FeSO}_4$  (standard) and  $\text{MeOH}$  (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 ( $\mu\text{M}$ ) 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  $0.70 \pm 0.02$  at 734 nm. Test samples (10  $\mu\text{L}$ ) at a final concentration  $1 \text{ mg mL}^{-1}$  were added in  
236 a 96 well plate with the radical solution (200  $\mu\text{L}$ ) 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  $\mu\text{M}$   
246 were used to prepare a standard curve for calibration. Hydrolysate test samples (100  $\mu\text{L}$ ) at a  
247 final concentration  $1 \text{ mg mL}^{-1}$  were diluted with methanol and incubated with 3.9 mL of 6  $\mu\text{M}$   
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<sup>-1</sup>). The EC<sub>50</sub> values were  
269 calculated and represent the concentration required that inhibits cell viability by 50%. Based  
270 on the EC<sub>50</sub> 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  $5 \text{ mg mL}^{-1}$  (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  $1 \text{ mg mL}^{-1}$  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<sup>-1</sup>, 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<sup>-1</sup> the hydrolysates significantly inhibited the production of the  
311 pro-inflammatory cytokines IL-6, IL-1 $\beta$  and TNF- $\alpha$ . 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<sup>-1</sup> 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  $\beta$ -casein (191–  
334 209) (LLYQEPVLGPVVRGPFPIIV) 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  $\alpha$ -CD3 and  $\alpha$ -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 H<sub>2</sub>O<sub>2</sub> treated HepG2 cells (Xie, Wang, Ao, & Li, 2013). Casein phosphopeptides  
359 (CPP), obtained by SGID, produced an increase in GSH and CAT activity in H<sub>2</sub>O<sub>2</sub> 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 H<sub>2</sub>O<sub>2</sub> (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 H<sub>2</sub>O<sub>2</sub> ( $p < 0.05$ ).



375 The rest of the hydrolysates and NaCN were not significantly different from the mean value  
376 obtained for H<sub>2</sub>O<sub>2</sub> treatment (Fig.1b).

377 This result may be due to the different mechanisms of action of the two oxidants.  
378 H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> (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  $\text{Fe}^{3+}$   
408 to  $\text{Fe}^{2+}$ . 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  $\text{H}_2\text{O}_2$ -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  $\text{H}_2\text{O}_2$ -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<sup>+</sup> assay, was then performed. However, the  
432 results showed that the activity of the hydrolysates against ABTS<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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 H<sub>2</sub>O<sub>2</sub> 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  $\mu\text{M}$ ) and (b) hydrogen peroxide (50  $\mu\text{M}$ ). 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  $\mu\text{mol TROLOX eq g}^{-1}$ ); (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.<sup>a</sup>

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*

<sup>a</sup> Cells were exposed to increasing concentrations (0.25–50 mg mL<sup>-1</sup>) 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. <sup>a</sup>

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*

<sup>a</sup> Cells were exposed to increasing concentrations (0.25–50 mg mL<sup>-1</sup>) 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**

EC<sub>50</sub> values of sodium casein (NaCN), NaCN non-cross-linked hydrolysate (Prolyve) and NaCN transglutaminase (TGase) cross-linked hydrolysates (TGase/Prolyve and Prolyve/TGase).<sup>a</sup>

Sample	EC <sub>50</sub> (mg mL <sup>-1</sup> )	
	Jurkat T	U937
NaCN	18.65 <sup>ac</sup>	31.55 <sup>a</sup>
Prolyve	30.55 <sup>b</sup>	13.96 <sup>b</sup>
TGase/Prolyve	16.02 <sup>ac</sup>	21.58 <sup>c</sup>
Prolyve/TGase	22.92 <sup>abc</sup>	14.94 <sup>bc</sup>

<sup>a</sup> Values are mean of at least three independent experiments. Different superscript letters denote significant difference ( $p < 0.05$ ) for each cell line. EC<sub>50</sub> 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. <sup>a</sup>

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

<sup>a</sup> 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).<sup>a</sup>

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

<sup>a</sup> Data are the mean of three independent experiments ± SE; none of the results was statistically significant compared with the untreated cells.

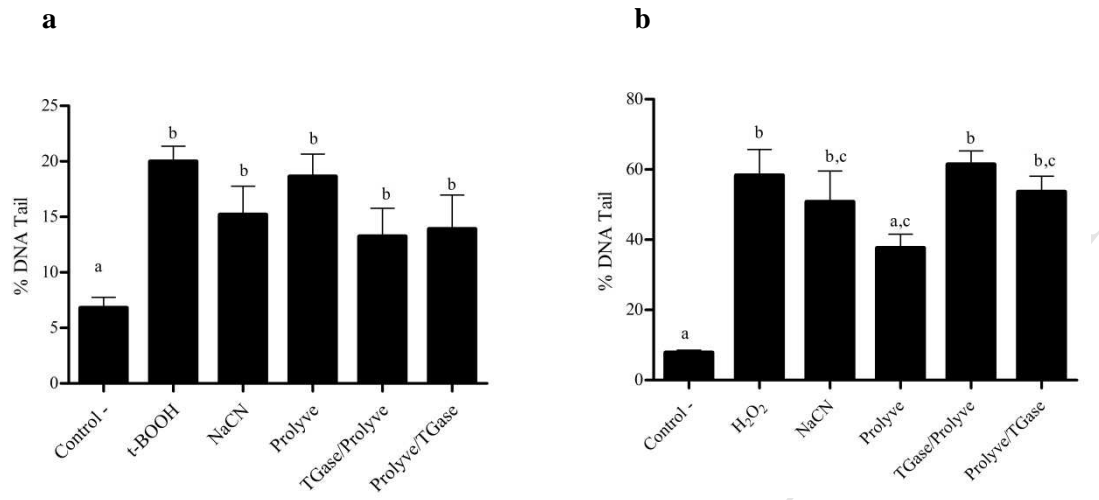


Figure 1.

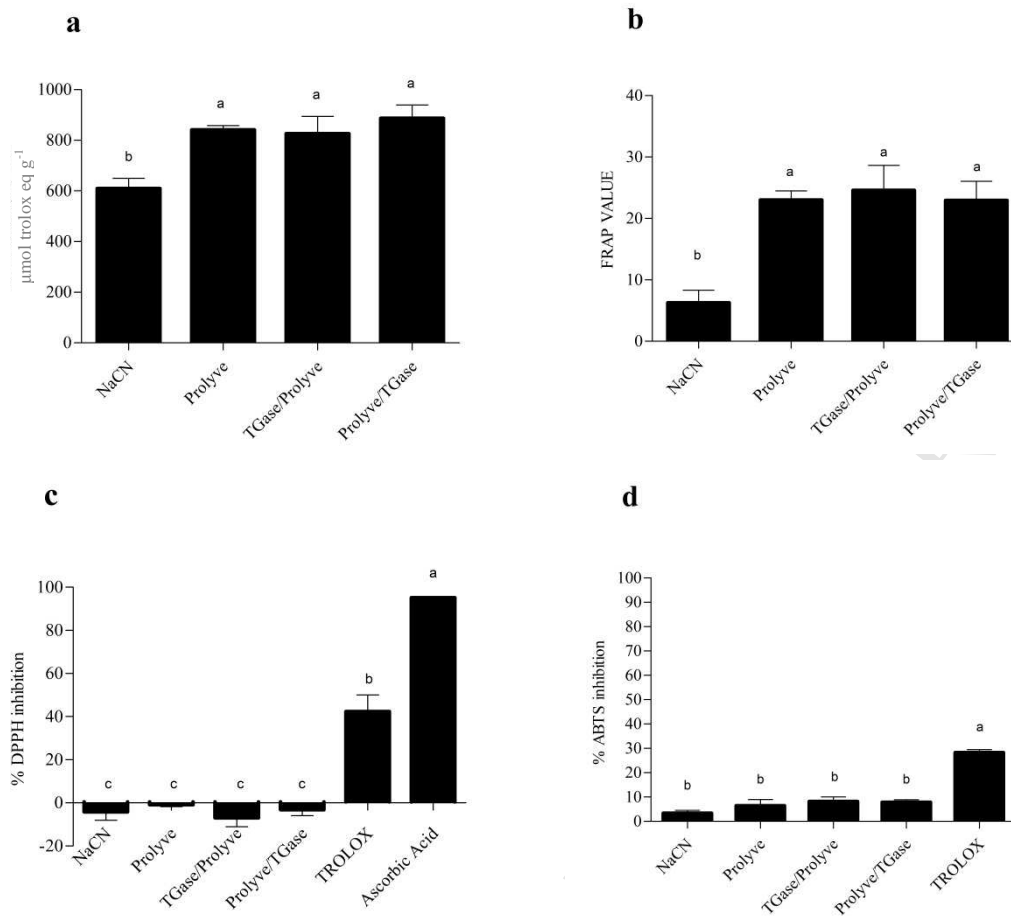


Figure 2.