



29 **Abstract**

30 Polyphenols are known for their antimicrobial activity, whilst both polyphenols and the globular protein beta-  
31 lactoglobulin (bLG) are suggested to have antioxidant properties and to promote cell proliferation. These are  
32 potentially useful properties to have as part of a tissue engineered construct, though it is unknown if they are  
33 retained when both compounds are used in combination. In this study, a range of different microbes and an  
34 osteoblast-like cell line (hFOB) were used to assess the combined effect of; (1) a green tea extract (GTE), rich in  
35 the polyphenol epigallocatechin gallate (EGCG), and (2) a whey protein isolate (WPI), rich in bLG. It was  
36 shown that approximately 20–48 % of the EGCG in GTE reacted with WPI. GTE inhibited the growth of gram-  
37 positive bacteria, an effect which was potentiated by the addition of WPI. GTE alone also significantly inhibited  
38 the growth of hFOB cells after 1, 4 and 7 days of culture. Alternatively, WPI significantly promoted hFOB cell  
39 growth in the absence of GTE and attenuated the effect of GTE at low concentrations (64 µg/ml), after 4 and 7  
40 days. Low concentrations of WPI (50 µg/ml) also promoted expression of the early osteogenic marker alkaline  
41 phosphatase (ALP) by hFOB cells, whereas GTE inhibited alkaline phosphatase ALP activity. Therefore, the  
42 antioxidant effects of GTE can be boosted by WPI, but it is not suitable to be used as part of a tissue-engineered  
43 construct due to its cytotoxic effects which negate any positive effect of WPI on cell proliferation.

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46 **List of Abbreviations**  
47  
48 ALP = alkaline phosphatase  
49 ANOVA = one-way analysis of variance  
50 ATCC = the American Type Culture Collection  
51 bLG = beta-lactoglobulin  
52 BOF = Bijzonder Onderzoeksfonds (Special Research Fund)  
53 CFU = colony forming units  
54 DAD = diode array detector  
55 ddH<sub>2</sub>O = sterile ultra-pure water  
56 DFG = Deutsche Forschungsgemeinschaft (German Research Foundation)  
57 DMEM = Dulbecco's modified Eagle medium  
58 DNA = deoxyribonucleic acid  
59 DPPH = 2,2-diphenyl-1-picrylhydrazyl  
60 EC = epicatechin  
61 ECG = epicatechingallate  
62 EGC = epigallocatechine  
63 EGCG = epigallocatechin gallate  
64 EUCAST = The European Committee on Antimicrobial Susceptibility Testing  
65 FRAP = ferric reducing ability of plasma  
66 FWO = Fonds voor Wetenschappelijk Onderzoek - Vlaanderen (Research Foundation - Flanders)  
67 GTE = green tea extract  
68 hFOB = human foetal osteoblast  
69 HPLC = high performance liquid chromatography  
70 MgCl<sub>2</sub> = magnesium chloride  
71 MH broth = Mueller-Hinton broth  
72 MIC = minimal inhibitory concentration  
73 MRSA = methicillin-resistant *Staphylococcus aureus*  
74 NaCl = sodium chlorid  
75 NaOH = sodium hydroxide  
76 PBS = phosphate buffered saline  
77 ROS = reactive oxygen species  
78 RPMI medium = Roswell Park Memorial Institute medium  
79 S.D. = standard deviation  
80 TEAC = Trolox equivalent antioxidant capacity  
81 TFA = trifluoroacetic acid  
82 WPI = whey protein isolate  
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85

86 **Introduction**

87

88 Antibacterial properties are highly desirable characteristics for bone regeneration materials, especially  
89 in oral and maxillofacial surgery due to the high microbial load of the oral cavity (George et al. 1994; Chen et  
90 al. 2010; Gosau et al. 2016). Due to the increasing occurrence of antibiotic-resistant bacteria, such as  
91 methicillin-resistant *Staphylococcus aureus* (MRSA) (Klein et al. 2016; Kavanagh et al. 2017), there is a  
92 pressing need to find alternative low cost antibacterial agents, to which there is little resistance.

93 Green tea extract (GTE) is known to be rich in polyphenols, which in turn possess antibacterial activity  
94 (Zhao et al. 2001; Gharib et al. 2013) (for a review see (Steinmann et al. 2013)). One major polyphenol of GTE  
95 is epigallocatechin gallate (EGCG), which shows antibacterial activity against MRSA (Gharib et al. 2013) and is  
96 alleged to stimulate the growth and differentiation of bone-forming cells (Vali et al. 2007; Jin et al. 2014). To  
97 improve stability of EGCG, it is commonly combined with carrier proteins, such as the globular protein beta-  
98 lactoglobulin (bLG) (Keppler et al. 2015). bLG is a major component of whey protein isolate (WPI), derived  
99 from milk. It consists of 162 amino acid residues, has a molecular weight of approximately 18.4 kDa and is  
100 known to be able to bind hydrophobic molecules (Kontopidis et al. 2004). Besides its application as a carrier  
101 protein, bLG has been reported to improve the proliferation of various mammalian cells (Moulti-Mati et al.  
102 1991; Mahmud et al. 2004; Gillespie et al. 2015). It is therefore hypothesised that bLG would also positively  
103 influence osteoblast growth, which would be desirable for bone regeneration applications.

104 Oxidative stress plays an important role in the immune response and has been identified as a  
105 pathological inducer in almost all organs (Wauquier et al. 2009). Despite its role in cell signaling, an increased  
106 level of oxidative stress can cause bone loss, leading to conditions such as osteoporosis, bone tumor  
107 development and inappropriate ingrowth of bone implants. Furthermore, increased levels of reactive oxygen  
108 species (ROS) have a supportive effect on osteoclasts, the cells responsible for bone resorption (Garrett et al.  
109 1990; Bai et al. 2005). Thus, antioxidant activity of compounds used during bone substitution could conceivably  
110 improve the stability of an implant. EGCG and bLG both have antioxidant activity, which make them excellent  
111 candidates as loading agents for bone tissue regeneration (Tong et al. 2000; Tobi et al. 2002; Mann et al. 2015).

112 Although there is some data available on the micro and cell biological effects of GTE, WPI and their  
113 components, the effects of GTE and WPI in combination remain unexplored. It was therefore hypothesized that  
114 WPI would influence GTE's antimicrobial and antioxidant activity, as well as its impact on osteoblast  
115 growth/differentiation. In the present study, chemical interaction of GTE and WPI was analyzed by  
116 centrifugation, ultrafiltration and subsequent high performance liquid chromatography (HPLC) analyses.  
117 Antimicrobial activity was tested against a range of bacteria (both Gram-positive and Gram-negative) and fungi,  
118 whilst effects on osteoblast proliferation and differentiation were tested using an osteoblastic cell line. Finally,  
119 antioxidant properties were investigated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) method. GTE has  
120 substantial antimicrobial and antioxidant capability and binds to WPI, which displayed positive effects on  
121 osteoblast activity.

122

123 **Materials and Methods**

124 *Materials*

125 Unless stated otherwise, all reagents were obtained from Sigma-Aldrich. WPI (BiPRO, Davisco Foods  
126 International, Inc., Eden Prairie, US) with 97.7% protein and 75% bLG in dry matter (according to  
127 specification) was used as previously described (Serfert et al. 2014; Keppler et al. 2017). GTE, > 95% (obtained  
128 from green tea leaves, catechins content>75%, EGCG>65%; according to specification) was purchased from  
129 Oskar Tropitzsch e. K. (Marktredwitz, Germany).

130

#### 131 *Material analysis*

##### 132 *Analysis of WPI*

133 The composition of the WPI was determined using a method described previously (Clawin-Radecker et al.  
134 2000; Keppler et al. 2014). Briefly, a HPLC HP 1100 system (Agilent Technology, Germany) equipped with a  
135 PLRP-S 300 Å 8 µm, 150 × 4.6 mm column (Polymer Laboratories, Varian, Inc.) and a diode array detector  
136 (DAD) at 205 nm wavelengths was used. Eluent A was 0.1% trifluoroacetic acid (TFA) in water and eluent B was  
137 0.1% TFA in acetonitrile. The following gradient was used: 0 min, 35% B; 1 min, 35% B; 8 min, 38% B; 16  
138 min, 42% B; 22 min, 46% B; 22.5 min, 100% B; 23 min, 100% B and 23.5–30 min, 35% B. Flow rate was 1  
139 ml/min, the injection volume was 20 µl and the column temperature was set to 40 °C. Standards of bovine  
140 serum albumin, alpha-lactalbumin and beta-lactoglobulin were run for identification.

##### 141 *Analysis of GTE*

142 GTE was analysed by HPLC (Agilent 1100, with a DAD at 280 nm wavelength) using a C18-Nucleodur Sphinx  
143 125/4 reversed phase column, 5 µm (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Eluent A consisted  
144 of water with 0.1 % formic acid, whilst eluent B was acetonitrile with 0.1 % formic acid. The following gradient  
145 was used: 0-1 min, 5 % B; 1-10 min, 95 % B; 10-10.5 min, 95 % B; 10.5 – 11 min, 5 % B and 11-12 min, 5 %  
146 B. Standards of the GTE components (EGCG, epigallocatechine (EGC), epicatechingallate (ECG) and  
147 epicatechin (EC)) were run to identify the respective signals in the GTE. Percentage composition of the  
148 components was assessed semiquantitatively, by comparing individual peak areas to the sum of all peak areas.

149

##### 150 *Binding studies*

151 For binding analysis, approximately 4 mg/ml WPI in water were weighed together with GTE in a concentration  
152 ratio of 1:0.08 g (w/w), 1:0.16 g (w/w), 1:0.32 g (w/w) and 1:0.64 g (w/w). Subsequently, samples were  
153 dissolved in demineralized water for 30min at room temperature with vigorous stirring. For control, solutions of  
154 GTE without WPI were prepared at a similar concentration. Vivaspin 2 centrifugal filtration devices  
155 (Hydrosart® regenerated cellulose membrane, 10 kDa), from Sartorius AG (Göttingen, Germany), were rinsed  
156 with 2ml demineralized water. Following this, 1ml of the respective sample solution (i.e. GTE with and without  
157 WPI) was centrifuged, using 4000\*g for 2 min at 20 °C - in a swinging-bucket rotor centrifuge. The filtrate and  
158 remaining retentate were discarded and replaced again by 1 ml of sample solution. After centrifugation, the  
159 filtrate was analysed via HPLC, using the aforementioned method for GTE analysis. As controls, uncentrifuged  
160 GTE samples were also analysed, to determine the amount of GTE lost in the membrane during filtration.

161

##### 162 *Antioxidant method (DPPH test)*

163 The radical scavenging activity of the different WPI/GTE complexes was analysed using a modification of the  
164 DPPH test described by Harbaum et al. (Harbaum et al. 2008). The DPPH assay was carried out by mixing 1 mL

165 of 0.3 mmol/L 2,2-diphenyl-1-picrylhydrazyl (DPPH radical) in ethanol with 1.95 mL of 25% aqueous ethanol,  
166 containing 0.1 M phosphate buffered saline (PBS) buffer. This gave a final 50% ethanolic solution. An initial  
167 reading was carried out at 516 nm (initial absorbance approximately 1.3). Subsequently, a 50  $\mu$ L sample (also  
168 dissolved in ethanol) was added, and the absorbance was measured after 10 minutes of reaction time. The  
169 antioxidative capacity was given as % inhibition: %inh =  $([A_0 - A_1]/A_0) * 100$ , where  $A_0$  is the initial absorbance  
170 of the standard and  $A_1$  is the absorbance of the sample.

171

#### 172 *Antibacterial studies*

173 *Staphylococcus aureus* ATCC 700699, ET199 and LMG10147, *Staphylococcus epidermidis* ET086 and *P.*  
174 *aeruginosa* ATCC9027 were cultured aerobically in Mueller-Hinton broth (MH; Oxoid, Basingstoke, England),  
175 at 37°C. *Propionibacterium acnes* LMG 16711 was cultured anaerobically in Reinforced clostridial medium  
176 (RCM; Oxoid), at 37°C. *Candida albicans* ATCC MYA-2876 was cultured aerobically in RPMI 1640 medium  
177 (Sigma-Aldrich, Diegem, Belgium), at 37°C.

178 Determination of the Minimal Inhibitory Concentration (MIC): MIC of GTE alone or in the presence of WPI  
179 (50 or 800  $\mu$ g/ml) was determined according to the EUCAST broth microdilution protocol, using flat-bottom  
180 96-well microtiter plates (TPP, Trasadingen, Switzerland). The inoculum was standardized to approximately  $5 \times$   
181  $10^5$  colony forming units (CFU)/ml. The concentration of GTE tested ranged from 0.5 - 1024  $\mu$ g/ml. The plates  
182 were incubated at 37°C for 24 h, and the optical density at 590 nm was determined using a multilabel microtiter  
183 plate reader (Envision; Perkin-Elmer LAS, Waltham, MA). MIC was recorded as the lowest concentration of  
184 GTE, alone or in combination with WPI, which displayed a similar optical density as that observed in inoculated  
185 and blank wells.

186

#### 187 *Cytocompatibility testing using fibroblasts*

188 Human fibroblasts were isolated from the gingiva of seven patients (4 female, aged 21, 25, 20 and 20 years and  
189 3 male, aged 36, 21 and 21 years) cultured in cell culture medium (Eagle's minimum essential Medium Alpha  
190 modification (Sigma-Aldrich GmbH, Hamburg, Germany), antibiotics (100 U x mL<sup>-1</sup> Penicillin, 100  $\mu$ g x mL<sup>-1</sup>  
191 Streptomycin, Biochrom, Berlin, Germany), 1 % Amphotericin (Biochrom), 15 % fetal bovine serum (FBS)  
192 (HyClone, Logan, Utah, USA) and 400 mmol x mL<sup>-1</sup> L-Glutamine) for 7 days, then medium was exchanged  
193 three times per week Ethical approval (number D444/10) was obtained from University Hospital Schleswig-  
194 Holstein, Campus Kiel, Germany. Fibroblasts in the third passage were pooled and  $5 \times 10^3$  were seeded in 96-  
195 well plates, i.e.  $1.56 \times 10^4$  cells/cm<sup>2</sup>. Cytocompatibility was assessed by incubating cells with medium  
196 containing 0, 50 or 800  $\mu$ g/ml WPI and 0, 64, 128, 256, 512, 1024, 2048 or 4096  $\mu$ g/ml GTE. Morphology was  
197 assessed by light microscopy after 7 days of culture. Medium was changed 3 times during this period.

198

#### 199 *Cytocompatibility testing using hFOB osteoblast-like cells*

200 An immortalized human foetal osteoblast (hFOB) cell line (hFOB 1.19; LGC Standards, USA) was used in this  
201 study, cultured in Dulbecco's modified Eagle medium (DMEM)/F12 (1:1) medium, supplemented with 10%  
202 FBS, 1% L-glutamine and 0.6% geneticin (all Gibco, Life Technologies, UK). Treatments were prepared at  
203 concentrations detailed in section 2.6, filter sterilised (0.22  $\mu$ m) before use and protected from light. Cells were

204 seeded at  $1 \times 10^5$  cells/cm<sup>2</sup> on 96-well sterile culture plates and given a 24 hour settling period before treatment.  
205 For all experimental conditions n=4.

206

#### 207 *hFOB proliferation*

208 Proliferation was measured by crystal violet staining at 1, 4 and 7 days. After removing culture medium by  
209 aspiration, cells were fixed with 100µl of 4% paraformaldehyde solution for 1 hr at room temperature, washed  
210 twice with ddH<sub>2</sub>O and left to air dry. Cell monolayers were then stained for 30 minutes at room temperature  
211 with 100µl of crystal violet solution per well (0.1% concentration), washed twice again in ddH<sub>2</sub>O and air dried.  
212 Finally, the dye was extracted from monolayers by the addition of 100µl of 1M acidified methanol. Absorbance  
213 was read at 585nm using a Tecan GENios microplate reader, and blanked using acidified methanol.

#### 214 *hFOB differentiation*

215 Alkaline phosphatase (ALP), an enzyme produced by maturing osteoblasts, was used as an indicator of cell  
216 differentiation. Briefly, cells were grown in complete medium supplemented with 10µM β-glycerophosphate  
217 and 50µM ascorbate-2-phosphate for 7, 14 or 21 days, before being washed with an alkaline buffer solution (5M  
218 NaCl, 1M Tris-Cl pH 9.5, 1M MgCl<sub>2</sub>). Cells were lysed by addition of 250µl of buffer - containing 0.2% Triton  
219 X-100 - and left to gently mix for 20 minutes on ice, before being stored at -80°C. Upon testing, 50 µl from each  
220 well was added to a test plate in duplicate. 200 µl of conditioned medium, consisting of alkaline buffer solution  
221 (Sigma) and p-nitrophenyl phosphate substrate was added to each well. Each test plate was then covered in foil  
222 and incubated for 30min at 37°C, allowing the coupled enzymatic reaction to proceed. The reaction was stopped  
223 by the addition of 50µl of stop solution (3M NaOH) and absorbance was read at 450nm. Finally, ALP readings  
224 were normalised to DNA concentration, determined via PicoGreen assay (in a method according to the  
225 manufacturer's protocol), to account for the effect of variances in cell proliferation.

226

#### 227 *Statistical analysis*

228 Each result set is presented as the mean ± S.D. (Standard Deviation) and has been tested for normality, after  
229 undergoing a logarithmic transformation. Differences between treatments were analysed using one-way analysis  
230 of variance (ANOVA) with post hoc Tukey's test. All statistics were conducted in SPSS version 19 (IBM,  
231 USA). Values of  $P \leq 0.05$  were considered significant.

232

## 233 **Results**

#### 234 *Extract composition and binding*

235 Catechine composition of the GTE (>95 % polyphenols) was analysed by HPLC using external standards for the  
236 main components EGCG, EGC (epigallocatechin), ECG (epicatechingallate) and EC (epicatechin). The main  
237 catechine of the GTE was EGCG (88.5±0.14%). Only minor fractions of other catechines were present in the  
238 order ECG > EGC > EC (i.e., 5.3 ±0.03%, 3.7 ±0.09% and 2.4±0.08%, respectively). WPI was also analysed by  
239 HPLC and consisted primarily of bLG AB (75.7±1.4%), with alpha-Lactalbumin (14.7±0.1%) and <4% bovine  
240 serum albumin (Keppler et al. 2017).

241 The combination of GTE and WPI resulted in a non-covalent interaction, which was analysed by  
242 ultrafiltration (Figure 1). Of the catechines present, only those which contained an esterified gallic acid, such as  
243 EGCG and ECG, showed a significant interaction with WPI. Furthermore, non-covalent binding of catechines to

244 WPI decreased with increasing GTE concentration (i.e., 43.7 % of the initial EGCG concentration in 64 µg/ml  
245 GTE was found to interact with 800 µg/ml WPI, whereas only 30.5 % EGCG reacted after addition of 512  
246 µg/ml GTE). ECG bound in a similar way to WPI, although with a slightly lower binding capacity (i.e., 45.3 %  
247 of the initial ECG concentration bound with WPI after addition of 64 µg/ml GTE, and 20.07 % after addition of  
248 512 µg/ml GTE). The loss of catechines due to unspecific membrane interactions and co-precipitation in the  
249 GTE during the ultrafiltration method was acceptable (<22 %).

250

#### 251 *Antioxidant and antibacterial effects of GTE and WPI*

252 In the DPPH test (Figure 2), the antioxidative capacity of WPI and GTE were tested by their capacity to  
253 scavenge the free DPPH radical. GTE alone exhibited a scavenging capacity of 20 and 28 % for 64 and 128  
254 µg/ml GTE, respectively. However, the addition of WPI had a significant negative effect on the radical  
255 scavenging capacity of GTE. For example, inclusion of 800 µg/ml WPI reduced inhibition to ~13 and ~24 % for  
256 64 and 128 µg/ml GTE, respectively. There was no radical scavenging effect of WPI alone.

257 For antibacterial activity, results of MIC (Table 1) determination revealed that in the absence of WPI  
258 the GTE concentrations required to inhibit growth of the bacteria ranged between 128-256 µg/ml. GTE hindered  
259 growth of gram-positive bacteria more strongly than growth of the gram-negative *P. aeruginosa*. Addition of  
260 WPI decreased the MIC of GTE towards the gram-positive bacteria *S. aureus* ATCC 700699 and *S. epidermidis*  
261 ET086, and in particular *P. acnes* LMG 16711. In contrast, WPI increased the MIC towards the gram-negative  
262 *P. aeruginosa* ATCC 9027. The growth of the yeast *C. albicans* ATCC MYA-2876 was not affected by GTE  
263 (MIC >1024 µg/ml, regardless of the addition of WPI).

264

#### 265 *Proliferation of fibroblasts and hFOB*

266 Fibroblasts cultured in the absence of GTE (Figure 3) showed a spindle-like morphology typical of healthy  
267 fibroblasts, as did those exposed to all concentrations of WPI (0, 50 and 800 µg/ml). However, increasing  
268 concentration of GTE appeared to promote the formation of round, apoptotic bodies, indicating the cells were  
269 preparing themselves for phagocytosis and were thus not viable. At a GTE concentration of 64 µg/ml, only a  
270 few apoptotic bodies were observed. At 128 µg/ml GTE larger numbers of dead cells were detected, whilst at  
271 256 µg/ml the majority of cells were dead. Higher GTE concentrations (data not shown) resulted in similar  
272 results as for 256 µg/ml GTE. Increasing WPI concentration to 50 or 800 µg/ml did not lead to an appreciable  
273 reduction in cell death. Hence, GTE concentrations above 128 µg/ml were excluded from further experiments.

274 For day 1 hFOB cultures, 64 and 128 µg/ml GTE reduced cell proliferation (Figure 4). This effect was  
275 not changed by the addition of WPI. At day 4, 64 and 128 µg/ml GTE reduced cell proliferation, but this  
276 reduction was attenuated by inclusion of 800 µg/ml WPI. At day 7, reduced cell proliferation was again  
277 observed with both 64 and 128 µg/ml GTE, though no attenuating effect of WPI was observed. At all GTE and  
278 WPI concentrations, the decrease in cell proliferation with GTE treatment was not a linear relationship.

279 WPI appears to promote small increases in cell proliferation, as in the absence of GTE 800 µg/ml WPI  
280 promoted cell proliferation at day 1 and 7. Furthermore, at 64 µg/ml GTE, proliferation was promoted by 800  
281 µg/ml WPI at day 1 and 4; whilst in the presence of 128 µg/ml GTE, 800 µg/ml WPI promoted proliferation at  
282 day 4 only. 50 µg/ml WPI had no significant effect at any time point or GTE concentration.



283 Images of hFOB cells stained using the crystal violet method (Figure 5) agree with those for fibroblasts  
284 (Figure 3), and support crystal violet assay results (Figure 4). For example, hFOB cells cultured in the absence of  
285 GTE formed healthy monolayers, though at a GTE concentration of 64 µg/ml markedly fewer cells were  
286 observed. Of these, some - but not all cells - displayed an elongated morphology, typical of good adhesion.  
287 However, at the highest GTE concentration of 128 µg/ml even fewer cells were observed, providing further  
288 evidence of the inhibitory effect of GTE on cell proliferation.

#### 289 290 *Differentiation of hFOB cells*

291 Only 0 and 64 µg/ml concentrations of GTE were used in differentiation experiments, as 128 µg/ml GTE had a  
292 detrimental effect on hFOB proliferation. Day 7 (Figure 6a) timepoint treatments showed the highest overall  
293 levels of alkaline phosphatase (ALP) activity. In WPI subgroup, 64 µg/ml GTE treatments showed significantly  
294 reduced ALP levels compared to those with no GTE. This trend is maintained at all timepoints, with the  
295 exception of the 0 µg/ml WPI subgroup at day 21, where ALP activity is very similar for both GTE treatments.  
296 WPI however shows a bell-shaped response, with 50 µg/ml concentrations causing increased ALP activity  
297 levels, compared to 0 and 800µg/ml treatment wells. These increases for the 50 µg/ml treatment group are  
298 significant at day 7, significant compared to the 800µg/ml treatment at day 14, and the 0 µg/ml treatments at day  
299 21.

#### 300 301 **Discussion**

302 This study aimed to determine the combined effects of GTE and WPI, focusing on traits that would be useful for  
303 bone regeneration materials – such as antimicrobial and antioxidant activity, as well as impacts on osteoblast  
304 growth/differentiation. Firstly, binding assay results showed that significant complexes between GTE and WPI  
305 occurred. This was expected, given that previous studies have reported the binding efficiency of whey proteins  
306 with green tea catechins (Keppler et al. 2015). For example, galloylated catechins, such as EGCG and ECG,  
307 were found to react most strongly with proteins, and exhibited good antioxidative capacity (Bohin et al. 2012).  
308 Due to the nature of the non-covalent reaction, the catechins bound to the protein are always in equilibrium with  
309 unbound catechins, explaining the reported 50% binding of this work.

310 GTE's high radical inhibition capacity was also expected, being mediated by its >95% polyphenol  
311 content, of which most take the form of EGCG (Supplementary Figure 1). Previous studies have shown EGCG  
312 and ECG to be two of the most potent radical scavenging flavonoids present in GTE (Salah et al. 1995; Hirano  
313 et al. 2001). However, the addition of WPI caused a masking effect on GTE scavenging activity, as evidenced  
314 by a decrease in its percentage inhibition. Such an effect on protein-bound polyphenols is probably mediated by  
315 hydrogen bonds, which occur between the hydroxyl groups of the catechins and the protein (Kanakis et al.  
316 2011). A similar masking effect was observed for caseins with green tea catechins using the Trolox equivalent  
317 antioxidant capacity (TEAC) test (Arts et al. 2001), and forBLG and EGCG complexes using the ferric reducing  
318 ability of plasma (FRAP) test (Zorilla et al. 2011). Interestingly, this masking effect on percentage inhibition  
319 observed in the present study amounted to a reduction of approximately 4 to 7%, after the addition of 50 and  
320 800 µg/ml WPI, respectively. However, the binding assay showed an interaction of approximately 50 % of the  
321 GTE. Therefore, it is likely that binding occurs in a way that leaves antioxidative groups of GTE partially  
322 available, even after interaction with the protein.

323 The main drawback of the DPPH test is the low solubility of DPPH in water, thereby introducing  
324 ethanol into the system, which could denature the WPI. It was reported that denatured whey proteins interact  
325 more readily with GTE catechines (Keppler et al. 2014), which could result in an overestimation of the masking  
326 effect for native WPI. It should be kept in mind that antioxidant assays used here (and in the literature) are  
327 optimized to stabilize the radical in solution, which requires either solvents like ethanol or methanol, or the  
328 addition of different salts. This will always influence the analysis of antioxidative capacity for protein-  
329 polyphenol complexes, because of the sensitivity of non-covalent binding reactions, which are likely altered  
330 even by the addition of salts or minor pH value changes.

331 GTE's anticipated antibacterial activity was confirmed in this study, with its inclusion at concentrations  
332 between 128-256 µg/ml inhibiting the growth of all bacteria tested. [This is in agreement with similar work](#)  
333 [within the field, whereby GTE extracts of various forms were shown to have diverse antibacterial activity – such](#)  
334 [as against \*S. aureus\*, \*S. epidermidis\*, \*P. acnes\* and \*P. aeruginosa\* \(Lee et al. 2009a; Sharma et al. 2012; Radji et](#)  
335 [al. 2013\). In the present study, addition of WPI facilitated GTE's antibacterial activity toward \*P. acnes\* LMG](#)  
336 [16711, with the MIC decreasing from 128 to 32 µg/ml \(50 µg/ml WPI\) and even 16 µg/ml \(800 µg/ml WPI\). A](#)  
337 [similar trend was also seen for \*S. aureus\* ATCC 700699 and \*S. epidermidis\* ET086, though MIC reduction was](#)  
338 [much less pronounced and was likely within the tests margin of error. For the Gram-negative Bacterium \*P.\*](#)  
339 [aeruginosa the opposite effect was seen, whereby addition of WPI gave rise to a drastic increase in MIC \(from](#)  
340 [256 to 1024 µg/ml\). Although the reasons behind the differences observed between Gram-positive and Gram-](#)  
341 [negative bacteria are unclear, the presence of a second \(outer\) membrane in Gram-negative bacteria may play an](#)  
342 [important role. It can be speculated that protein-bound catechins are less able to interact with peptidoglycan in](#)  
343 [the bacterial cell wall, making it difficult for these complexes to cross the outer membrane. EGCG also has a](#)  
344 [negative charge, which might be a reason for its lower affinity to Gram-negative bacteria \(Yoda et al. 2004\).](#)  
345 [Furthermore, it should be noted that previous work showed \*P. aeruginosa\* to have a MIC twice that of \*S. aureus\*](#)  
346 [\(800 compared to 400 µg/ml\) \(Radji et al. 2013\), which may partly explain the increase in MIC seen after WPI](#)  
347 [inclusion. Finally, the fungus, \*C. albicans\*, did not respond at all to GTE treatment \(MIC >1024 µg/ml\).](#)  
348 [Previously, it was reported \(Hirasawa and Takada 2004\) that EGCG had an anti-\*C. albicans\* effect, which](#)  
349 [increased with increasing pH and concentration. In our study, pH was 7.4 and the concentration was 1000x](#)  
350 [lower, which may explain the lack of response seen. However, the mechanism of GTE's anti-\*C. albicans\* effect](#)  
351 [remains unclear.](#)

352 Good antibacterial and antioxidant capacity are both desirable characteristics for regeneration  
353 materials, helping support a healthy healing site after bone damage. However, cell response also needs to be  
354 determined, to ensure cytocompatibility before clinical application. Fibroblast images (figure 3) helped to give a  
355 qualitative insight into GTE and WPI's effect, showing WPI to have no obvious impact on cell adhesion or  
356 morphology. However, increasing GTE concentration caused significant changes in cell morphology,  
357 attachment and increased mortality. [This is similar to findings of another study, whereby both normal and keloid](#)  
358 [fibroblasts were shown to decrease proliferation in a dose-dependent manner with EGCG treatment \(Park et al.](#)  
359 [2008\). Returning to the present study, fibroblast findings were also mirrored in proliferation results of an](#)  
360 [osteoblast-like cell line \(hFOB 1.19\), whereby 800 µg/ml WPI increased cell growth. Alternatively, GTE](#)  
361 [inclusion reduced proliferation, particularly at later time intervals, and this was only partially mitigated by the](#)  
362 [addition of WPI.](#)

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363 Previous work reports that WPI, especially its component bLG, has a mitotic effect on different cell  
364 types, which would explain its stimulative effect on hFOB proliferation. [For instance, Gillespie et al. reported](#)  
365 [that bLG \(312.5-2500 µg/ml\) improved the proliferation of enteroendocrine cells \(Gillespie et al. 2015\), whilst](#)  
366 [another study investigated mouse spleen resting cells. Here, a proliferative effect was found in the concentration](#)  
367 [range 50-500 µg/ml, over 12-96 hours \(Mahmud et al. 2004\).](#) For GTE polyphenols, the literature often reports a  
368 [positive effect on bone metabolism, such as increased osteoblast maturation and production of mineralised](#)  
369 [material \(Ko et al. 2009\).](#) Our opposite finding could be due to the different composition of catechins used in  
370 [this study, as work by Ko et al. \(Ko et al. 2009\) did not include EGCG specifically. Alternatively, it may](#)  
371 [indicate that even the minimum GTE concentration used in this work was too high, causing suppressive effects](#)  
372 [on cell activity. In support of this, other similar studies using osteoblast-like cells tended to use lower](#)  
373 [concentrations, compared to the 64 and 128 µg/ml doses of the present study. For example, rat mesenchymal](#)  
374 [stem cells \(Ko et al. 2011\), Saos-2 \(Nash and Ward 2016\) and human osteoblast cells \(Vester et al. 2014\) all](#)  
375 [showed increased levels of cell differentiation and limited cytotoxicity when treated with GTE concentrations of](#)  
376 [around 10 µg/ml \(a maximum of 50 and minimum of 0.01 µg/ml\). Another similar study, testing EGCG,](#)  
377 [demonstrated no toxic effect at 10 µg/ml \(Lee et al. 2009b\); and whilst higher concentrations are occasionally](#)  
378 [tested, exposure times are generally shorter. For example, rat calvarial osteoblasts were treated with green tea](#)  
379 [polyphenols at a 200 µg/ml dose, to prevent alterations upon exposure to H<sub>2</sub>O<sub>2</sub>; though only for a 1 hour period](#)  
380 [\(Park et al. 2003\).](#) Returning to the present study, GTE's inclusion also reduced bLG bioactivity, or  
381 bioavailability, which most likely resulted from binding between the two extracts.

382 The final indicator of cell activity investigated was ALP, expressed during osteoblast maturation,  
383 which is considered an early marker of osteogenic differentiation (Setzer et al. 2009). Though it was evaluated  
384 on days 7, 14 and 21, the highest ALP activity was measured on day 7, in the 50 µg/ml WPI treatment group.  
385 The consistently high ALP activity in this treatment, compared to 0 and 800 µg/ml, indicates WPI has an  
386 optimal concentration able to stimulate hFOB differentiation. On the other hand, GTE caused a decrease in ALP  
387 activity, often reducing the activity levels by 50% or more in wells with no GTE added. This is in agreement  
388 with a study (Yamaguchi and Ma 2001) concerning the effect of polyphenols on ALP activity in rat femoral  
389 tissues, which concluded that EGCG (0.1 mM) significantly inhibited ALP activity. Furthermore, the current  
390 study's results appear to support a previously reported interaction between ALP and polyphenols from the  
391 seaweed-derived nutritional supplement Seanol® (Douglas et al. 2016). Here, a similar concentration of  
392 polyphenols were used in supplements, which were shown to have reduced release rates during ALP-mediated  
393 mineralisation. [However, it is important to note there are also several studies where treatment with GTE](#)  
394 [increased osteoblast differentiation, such as with the aforementioned work using rat mesenchymal stem cells](#)  
395 [\(Ko et al. 2011\), Saos-2 \(Nash and Ward 2016\), and human osteoblast cells \(Vester et al. 2014\). These cells](#)  
396 [showed increased levels of many differentiation indicators, including ALP activity, mineralisation levels and](#)  
397 [gene expression for proteins including sclerostin, osteopontin and osteocalcin. The contrasting reports of GTE](#)  
398 [both inhibiting and promoting cell differentiation are likely a reflection of the overall concentration and](#)  
399 [polyphenol content of each extract tested.](#) Finally, in terms of the present study's interaction effects of GTE and  
400 WPI, GTE again reduced WPI bioactivity. However, the presence of 50 or 800 µg/ml WPI did not obviously  
401 mitigate the negative effect of 64 µg/ml GTE, indicating more WPI binding is needed to promote hFOB  
402 differentiation in these treatments.

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403 Cell based assay results are arguably the most important when determining a compound or extract's  
404 suitability for different applications, such as tissue engineering. GTE's cytotoxicity, whilst slightly mitigated by  
405 WPI, makes it unsuitable for inclusion at sites undergoing skeletal regeneration, as it would likely limit  
406 osteoblast activity. One related limitation of this work was the minimum GTE concentration tested, of 64 µg/ml.  
407 Lower concentrations may have preserved GTE's antimicrobial and antioxidant capacity, whilst also limiting  
408 cytotoxicity – especially with WPI inclusion. Similarly, further processing of GTE could have been conducted,  
409 to create GTE treatments with different polyphenol compositions and potentially better cytocompatibility.

410 This work demonstrated the affinity of WPI for components of GTE, and the formation of WPI/GTE  
411 complexes. GTE displayed excellent antioxidative capacity, which was not significantly affected by addition of  
412 WPI; though some masking effect is possible. Furthermore, GTE showed antimicrobial activity against gram-  
413 positive and gram-negative bacteria, with WPI inclusion potentiating this effect on several types of gram-  
414 positive bacteria. However, with cells GTE showed cytotoxic and suppressive effects on both fibroblasts and  
415 hFOB, especially at concentrations of 128 µg/ml and above. WPI alone though enhanced hFOB proliferation  
416 and attenuated the suppressive effect of GTE at 64 µg/ml, to a certain extent. For hFOB differentiation, WPI  
417 significantly stimulated ALP activity at a 50 µg/ml concentration, though was less able to attenuate effects of  
418 GTE inclusion. These results therefore show that WPI and GTE both have useful properties, but the cytotoxicity  
419 of GTE makes it unsuitable for inclusion within a tissue engineered construct.

420

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426

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555  
556

557 **Figure captions**

558

559 Figure 1: GTE compounds that were bound to 800 µg/ml WPI, as a function of the GTE [µg/ml] concentration.  
560 Abbreviations: EGC, Epigallocatechin; EC, epicatechin; EGCG, epigallocatechin gallate, ECG,  
561 epicatechingallate; GTE, green tea extract

562

563 Figure 2: Antioxidative capacity (DPPH test) of different concentrations of GTE, with or without addition of  
564 800 or 50 µg/ml WPI. All values are listed as mean ± standard deviation (n=3). \*: p < 0.05

565

566 Figure 3: Images of fibroblasts cultured in cell culture medium containing different concentrations of GTE  
567 (epigallocatechin gallate-rich extract) and WPI (beta-lactoglobulin-rich extract) after 7 d. Scale bar = 100 µm in  
568 all cases.

569

570 Figure 4: Data from day 1 (a), 4 (b) and 7 (c) crystal violet assay conducted on hFOB cells. Results are  
571 presented as the mean optical density values for each treatment, (n=4, +/- SD). \* indicates a significant  
572 difference (p<0.05) between the treatment and the 0µg/ml GTE value (within each WPI treatment group). #  
573 indicates a significant difference (p<0.05) between the treatment and the respective value for WPI = 0 µg/ml.

574

575 Figure 5: Images taken of hFOBs using an ISH500 camera attached to an OLYMPUS SZX10 microscope at 2x  
576 zoom. hFOB cells were fixed and stained with crystal violet 7 days after initial treatment. Scale bar = 1 mm in  
577 all cases.

578

579 Figure 6: data from an ALP assay conducted on hFOBs. Cells were given a 24 hour attachment period before  
580 being treated with solutions containing different concentrations of GTE and WPI. The assay was conducted on  
581 day 7 (a), day 14 (b) and day 21 (c) timepoints. Each ALP reading was normalized to the DNA concentration of  
582 the same well, calculated via PicoGreen assay. Results are presented as the mean +/- standard deviation (n=4).  
583 \*p<0.05 for WPI treatments at 64 µg/ml GTE, compared to WPI treatments of the same concentration at 0µg/ml  
584 GTE. #p<0.05 for WPI treatment comparisons at 0 µg/ml GTE.