

Materials Science & Engineering C

Phloroglucinol-enhanced whey protein isolate hydrogels with antimicrobial activity for tissue engineering --Manuscript Draft--

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Corresponding Author:	Maria Chatzinikolaidou University of Crete Heraklio, Greece
First Author:	Varvara Platania
Order of Authors:	Varvara Platania Timothy E.L. Douglas Mikhajlo K. Zubko, Dr. Danny Ward Krzysztof Pietryga Maria Chatzinikolaidou
Abstract:	<p>Aging populations in developed countries will increase the demand for implantable materials to support tissue regeneration. Whey Protein Isolate (WPI), derived from dairy industry by-products, can be processed into hydrogels with the following desirable properties for applications in tissue engineering: (i) ability to support adhesion and growth of cells; (ii) ease of sterilization by autoclaving and (iii) ease of incorporation of poorly water-soluble drugs with antimicrobial activity, such as phloroglucinol (PG), the fundamental phenolic subunit of marine polyphenols. In this study, WPI hydrogels were enriched with PG at concentrations between 0 and 20% w/v. PG solubilization in WPI hydrogels is far higher than in water. Enrichment with PG did not adversely affect mechanical properties, and endowed antimicrobial activity against a range of bacteria which occur in healthcare-associated infections (HAI). WPI-PG hydrogels supported the growth of, and collagen production by human dental pulp stem cells and - to a lesser extent - of osteosarcoma-derived MG-63 cells. In summary, enrichment of WPI with PG may be a promising strategy to prevent microbial contamination while still promoting stem cell attachment and growth.</p>
Suggested Reviewers:	Håvard Jostein Haugen, Dr. Professor h.j.haugen@odont.uio.no Justyna Kozłowska, Dr. Nicolaus Copernicus University in Torun Faculty of Chemistry: Uniwersytet Mikołaja Kopernika w Toruniu Wydział Chemii justynak@umk.pl Anastasiya Soloveva, Dr. Head of Lab, Novosibirsk Science Center of the Siberian Branch of the Russian Academy of Sciences: Novosibirskij Naucznyj Centr Sibirskogo otdelenia Rossijskoj akademii nauk solovevao@gmail.com Ioannis Chronakis, Dr. Professor, Technical University of Denmark National Food Institute: Danmarks Tekniske Universitet Fodevareinstituttet ioach@food.dtu.dk
Response to Reviewers:	

ΠΑΝΕΠΙΣΤΗΜΙΟ ΚΡΗΤΗΣ



UNIVERSITY OF CRETE

ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ &
ΤΕΧΝΟΛΟΓΙΑΣ ΥΛΙΚΩΝ

DEPARTMENT OF MATERIALS SCIENCE &
TECHNOLOGY

Dr. Maria Chatzinikolaidou, FBSE
Associate Professor of Biomaterials in Bioengineering
Department of Materials Science and Technology
Laboratory of Biomaterials in Tissue Engineering
University Campus Voutes, University of Crete, GR-70013 Heraklion, Greece
<https://www.materials.uoc.gr/el/general/personnel/mchatzin.html>
and collaborating Faculty Member at
Institute of Electronic Structure and Laser (IESL), Foundation for Research and Technology Hellas (FORTH)
N. Plastira 100, GR-71110 Heraklion, Greece
Phone: + 30 2810 394276 (office), 394277 (lab)
Email: mchatzin@materials.uoc.gr; mchatzin@iesl.forth.gr

Heraklion, 10 August 2021

Dear Editor, dear Prof. Dalby,

I would like to submit the revised manuscript entitled 'Phloroglucinol-enhanced whey protein isolate hydrogels with antimicrobial activity for tissue engineering' by V. Platania, T.E.L. Douglas, M.K. Zubko, D. Ward, K. Pietryga, M. Chatzinikolaidou.

We would like to thank all the Reviewers for their valuable comments helping us improving our manuscript. In the revised manuscript version we have addressed all Reviewers' comments in track change mode.

Thank you for your attention to our revised manuscript.

Sincerely

On behalf of all co-authors

Associate Professor Maria Chatzinikolaidou

Reviewer #1:

This manuscript investigated alendronate-tethered hydrogels for bone regeneration through a simple mixing of an alendronate-substituted alginate solution (Alg-AlenCa) and a sodium alginate solution (NaAlg), without an additional crosslinking process. The produced Alg-Alen hydrogels exhibited significantly improved mechanical and biological properties, compared with pure alginate hydrogels crosslinked with CaCl₂ solution. In particular, the best performance in mechanical properties and osteoconductivity was observed in the Alg-Alen 60 hydrogel. However, we still have following comments to be addressed by the authors:

- 1' In the schematic of the article, the red triangle on the Alg-AlenCa chain should not only be expressed as PO₃Ca, but also as i-COOCa.
- 2' Will ALG-Alen turn into a gel after contact with calcium ion solution? How did the author control it to maintain the solution state?
- 3' The author did not explain the reason for the shortening of gelation time with the increase of ALG-Alen content.
- 4' Figure 4 showed that the compressive strength of the control group was higher than that of the ALG-Alen 40 group, but the Page 11 of the article showed that the compressive strength of the ALG-Alen 40 group is 1.2 times that of the control group. why?
- 5' The author should do some experiment of osteogenesis of hydrogel in animals.

[Response: These comments refer to another manuscript, and we therefore skip them.](#)

Reviewer #2:

This work presented by Platania and co-workers was aimed to develop a whey protein isolate hydrogel enhanced with phloroglucinol. They characterized the morphology, mechanical properties, antimicrobial activity and biocompatibility of the composite. The concept is rather interesting, however, the current conclusions are lacking of critical experimental support.

For the reasons outlined below, I recommend that the article not be published without the following revisions.

Major:

- The authors proposed the material to be used for bone tissue engineering, while no osteogenic related experiments were carried out to support their title of the manuscript.

[Response:](#)

We thank the Reviewer for this comment. The data presented in this study including the quantification of total collagen production for the formation of a healthy extracellular matrix have merit for tissue engineering applications in general, therefore, we have removed the word 'bone' from the title, and we think that an *in vitro* and *in vivo* study of osteogenesis-related markers is not necessary. In addition, we have added a sentence in the discussion 'The exploration of the use of WPI-PG hydrogels in bone tissue engineering would necessitate more experimental data on the *in vitro* and *in vivo* osteogenic potential of these materials.'

- Typically, the bone defect happens to be an **irregular shape**, and the advantage of

hydrogels is the injectability to fill the defect. Herein, the developed PG-hydrogel is pre-formed. The authors should **discuss** the rationale for the design.

Response:

We thank the Reviewer for this comment. The reviewer is correct to note that one advantage of using certain hydrogels is injectability, allowing complete filling of the defect in principle. However, pre-formed hydrogels have also been considered as scaffolds to promote bone regeneration. Hydrogels have advantages over other materials commonly used for bone regeneration, such as ceramic materials, namely the lower brittleness and greater ease of incorporation of biologically active substances, which is demonstrated clearly by our study. This study is intended as a first study to prove the principle of using WPI hydrogels loaded with PG for tissue regeneration. Future plans would focus on the optimization of the scaffold, for instance by incorporation of a ceramic phase, to combine the advantages of hydrogels and ceramic materials for bone tissue engineering.

To address the Reviewer's comment more fully, we have added a paragraph to the introduction to explain the rationale for the design and another paragraph to the discussion.

- Figure 1, bubbles could be observed in the macroscopic images of the samples. Any effect because of the bubbles?

Response:

We thank the Reviewer for this comment. We do not expect an effect due to the bubbles, which are not present in the WPI solution, but appear to form as a result of steam sterilization. We suspect that the "bubbles" are hence "steam-filled" during formation and "vacuum-filled" after cooling to room temperature. Hence, we do not expect any effect.

- Figure 3, the 20% PG might significantly affect the mechanical properties as the authors stated. Therefore, the data might confuse the readers. In addition, the authors just simply described the results without in-depth discussion. For example, "increasing the PG concentration to 5% resulted in the compressive strength decreasing by 1.35 MPa to a mean of 2.91 MPa." What are the possible causes? Or mechanism?

Response:

We thank the Reviewer for this comment. Increasing the PG concentration to 5% did decrease the compressive strength, but not significantly - in the text we incorrectly stated that the difference was statistically significant - this has now been corrected to state that the difference was statistically insignificant. We have also added some text to the discussion to speculate about possible mechanisms, while pointing out that the discussion must remain speculative due to the limited data available.

- Figure 4, B and D, the authors normalized the data from A and C. However, for the TCPS groups, all the data were normalized to 100%. It did not represent the actual results. Plus, the **statistical method** is incorrect with One-way ANOVA. Time and concentration are two factors.

Response:

We appreciate the Reviewer comments. Cell proliferation is defined as the cellular growth rate or as the quantified value for the daughter cell population [1]. % Viability is the

quantification of the number of living cells and is usually expressed as a percentage of the control [2, 3]. In our case, tissue culture polystyrene (TCPS) coated wells were used as positive control.

So, for the quantification of % viability:

$$\frac{\text{Sample Absorbance } 570/600\text{nm}}{\text{Control Absorbance } 570/600\text{nm}} \times 100\%$$

for TCPS this ratio = 1, and %viability = 100

Indeed, the experimental data were analyzed using two-way ANOVA followed by Tukey's multiple comparisons test among groups. We have corrected the text in subsection 2.9 accordingly.

- Figure 5 and 6, the cells on the surface are rather spherical, while, good attachment should be spread and **spindle shape**.

Response:

The authors agree with the Reviewer and corrected the text related to the SEM results (section 3.4) to address this comment.

The sentence reads now: "Images at days 4 and 7 showed cell spreading for increased PG concentrations in WPI-PG-10% and WPI-PG-20% hydrogels with DPSCs, however without displaying their characteristic spindle shape morphology until day 7."

- Figure 7, the authors quantified the collagen. Which **type of collagens**? As is known MG63 can produce type III collagen. This experiment did not bring too much meaningful information.

Response:

We thank the Reviewer for this comment. We added more details about the Sirius red assay in materials and methods section. This modified Sirius red assay was used in order to stain total collagen produced in cell culture as an indicator of (extracellular matrix) ECM formation.

Collagen quantification results showed significantly higher total collagen production found in DPSCs cultures on WPI-PG hydrogels compared to the MG-63 cells, which is in line with the cell-cell interactions observed for DPSCs by means of SEM, suggesting that WPI-PG hydrogels may have supported the ECM formation when seeded with DPSCs.

- "no **cytotoxic** effect was observed on days 2 and 4 for both cell types that displayed high viability rates compared to the TCPS control", according to the data, this is not correct.

Response:

Thank you for this comment that helps us clarifying this issue. Regarding the cell viability assessment of the DPSCs and MG-63 cells on the WPI-PG hydrogels and according to ISO 10993-5 (2009) (Reduction of cell viability by more than 30 % is considered a cytotoxic effect), no cytotoxic effect was observed on days 2 and 4 for both cell types that displayed viability rates from 87-145% compared to the TCPS control, with higher values on WPI-PG-2.5% and WPI-PG-5% hydrogels on day 2. On day 7, the cell viability for DPSCs ranged from 60% to 73% compared to the control, while MG-63 cells presented viability values from 40%

to 65%. Increased PG concentrations led to an inhibition of the MG-63 proliferation, consistent with the previously reported anti-cancer activity of PG [4, 5].

- Comprehensive experiments are lacking in this manuscript. **Osteogenic experiments *in vitro* and *in vivo*** are suggested.

Response:

We thank the Reviewer for this comment. The data presented in this study including the quantification of total collagen production for the formation of a healthy extracellular matrix are of merit for tissue engineering applications in general, hence, we have removed the word 'bone' from the title, and we think that an *in vitro* and *in vivo* study of osteogenesis-related markers is not necessary. In addition, we have added a sentence in the discussion 'The exploration of the use of WPI-PG hydrogels in bone tissue engineering would necessitate more experimental data on the *in vitro* and *in vivo* osteogenic potential of these materials.'

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Reviewer #3:

1. I would suggest the authors to provide some more details on the advantages of hydrogels over scaffolds to treat critical bone defects, e.g. of scaffolds reported can be found here doi.org/10.1016/j.colsurfb.2017.09.004

Response:

We thank the reviewer for this comment. We added a paragraph in the introduction section, providing more details about the advantages of hydrogels over scaffolds to treat critical bone defects together with the suggested by the reviewer reference.

2. How efficient are the developed hydrogels regarding mid- and long term antibacterial protection.

Response:

The reviewer raises an interesting question: ideally, an antibacterial biomaterial would exhibit a burst release to prevent microbial colonization and help the recipient's cells win the "race to the surface", while also showing a prolonged release profile to prevent later microbial colonization. For this, studies of PG release are necessary in future work. We have added text in the discussion to mention the point raised by the reviewer.

3. Are the level of compression strength and Young's modulus below 20 and 10 MPa respectively, sufficient for load-bearing applications and critical bone size defect healing?

Response:

Materials for bone healing are generally not load-bearing unless they are permanent metallic implants, e.g. hip and knee endoprostheses, dental implants for tooth replacement. In critical defects, mechanical stabilization is not provided by the biomaterial for bone regeneration itself, but by external stabilizers.

4. Did the authors observe any effect that certain level of antibacterial component also deteriorated cell behavior?

Response:

It was observed that at proliferation and viability of MG63 cells seemed to be lower at the highest concentrations of PG (10%, 20%), which also showed the highest antimicrobial activity. It is possible that one must strike a balance between antimicrobial activity and cell proliferation and viability. Interestingly the reduction in proliferation and viability was not as pronounced for DPSCs. One may speculate that MG63 cells, being osteosarcoma-derived, are more susceptible to possible anticancer effects of PG, which have been reported previously for phlorotannins, of which PG is the fundamental subunit. We have added an extra paragraph to the discussion to address the reviewer's question.

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Reviewer #4:

This is a very valuable piece of work dedicated to development of scaffolds made of whey protein isolate and phloroglucinol characterised with multifunctional properties: support of osteogenic cells adhesion, proliferation and differentiation as well as antibacterial properties towards several pathogenic strains.

Prior to publication I recommend the Authors to:

- 1) unify the term Young's modulus (not Young modulus) all over the text, e.g. in page 8, page 9 (Fig. 3 and Fig. 3 caption), page 14 and 15.
- 2) introduce scale bar of better visibility in SEM pictures, i.e. Fig 2, Fig 5, Fig 6.

Response:

We appreciate these comments and we followed your advices.

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Reviewer #5

The manuscript entitled "Phloroglucinol-enhanced whey protein isolate hydrogels with antimicrobial activity for bone tissue engineering" is well written, logically structured and fits in the scope of the journal. I recommend the acceptance of the manuscript after minor corrections (mentioned online and below).

1. The English language and grammar used in the present manuscript is generally good. However, there are still of instances where mistakes, and unnecessary repetitions are present.

- Some grammatical mistakes e.g. („require" instead of requires, „a human osteosarcoma" instead of human osteosarcoma, „species included into the recently published list" instead of species included in the recently published list.

- Latin-derived words should be written in italic e.g. *in vivo*.

- In some places space is missing e.g. „...proliferation and osteogenic differentiation.In this paper...".

- The avoidance of repetition and the use of synonyms would be appreciated.
- Unification of the presentation of the units would be appreciated.
- There are mixed British & American spelling variants e.g. characterised vs characterized

Response:

We appreciate these comments and we followed your advices.

2. Introduction:

- The issues with HAI should be mentioned before the introduction of WPI and polyphenols.

Response:

We appreciate these comments and we followed your advices.

- To the sentence „Polyphenols have been implicated as components of antimicrobial hydrogels" any literature should be provided.

Response:

To address this suggestion, to the sentence "Polyphenols have been implicated as components of antimicrobial hydrogels" any literature should be provided", we included two references [6, 7].

3. Materials and Methods

- How many samples were used during mechanical tests? 9? It should be mentioned in methods.

Response:

We thank the reviewer for pointing this out. 8 samples were used per group. We have added this information to the methods section.

- What was the reason for using two different media during the cell studies (α -MEM or DMEM)?

Response:

For the two different cell types, different culture media are recommended. Specifically, DPSCs were established after generous donation by Prof. A. Bakopoulou at the School of Dentistry at the Aristotle University of Thessaloniki, based on a previously described protocol [8], and were cultured in minimum essential media α -MEM (Invitrogen) supplemented with 15% fetal bovine serum (FBS, Invitrogen), 100 mM L-ascorbic acid phosphate (Sigma-Aldrich, Steinheim, Germany), 100 units/ml of penicillin, 100 mg/ml of streptomycin and 1% amphotericin as previously described by the same team [9-11]. The osteosarcoma cell line MG-63 was purchased from Sigma (USA) and cultivated in DMEM containing 100 units/ml of penicillin, 100 mg/ml of streptomycin and 1% amphotericin (all from Gibco), supplemented with 10% fetal bovine serum (FBS) according to bibliography [12-14].

- Please unify the cell count $20 \times 10^3 = 2 \times 10^4$

Response:

We appreciate this comment and we modified accordingly.

- „Iso-sensitest agar plates (sometimes nutrient agar plates)" what does it mean?

Response:

For sensitivity tests, the samples were placed on the surface of plates containing 25 ml of Iso-sensitest agar (Oxoid CM0471) or (in some experiments) nutrient agar. Before placing the samples, the agar plates were swabbed with microbial suspensions".

- Maybe section „2.2 Sample preparation for scanning electron microscopy" could be named as 2.2 Surface topography studies.

Response:

We appreciate this comment and we modified accordingly.

4. Results

- „...are dried in preparation for SEM." could be changed to "during preparation for SEM studies"

Response:

We appreciate this comment and we modified accordingly.

- „It is important to note that, due to high friction caused by the nature of the samples, the WPI hydrogels supplemented with 20% PG became stuck in the jaws of the Zwick testing machine, meaning values for strength, modulus and strain at break may have been overestimated." All WPI-PG-20% samples behaved similarly? If yes, can Authors suggest a modification of the method?

Response:

We thank the reviewer for this comment. We have added a sentence to the discussion to describe a possible improvement of the method.

- Compressive strength for WPI-PG-20% was omitted in the comments.

Response:

We have added text to the discussion of the mechanical properties and to the results section 3.2.1.

- Authors have written „A statistically significant increase of 0.43 MPa was observed when PG concentration was increased from 5% to 10%." indication of the described significance on

the graph would be appreciated.

Response:

We thank the author for pointing out this inaccuracy. We meant “statistically insignificant”; this has now been corrected.

- DPSCs displayed a viability increase from day 4 to day 7 in all samples, with the WPI-PG-10% and WPI-PG-20% hydrogels demonstrating similar cell viability to TCPS control (Fig.4 B,D)." On the graph it is the opposite.

Response:

Thank you for this comment that helps us clarifying this issue. In terms of cell viability, we referred to metabolic activity expressed as absorbance value. We modified this sentence accordingly:

“DPSCs displayed increased metabolic activity, expressed as absorbance value, from day 4 to day 7 in all samples, with the WPI-PG-10% and WPI-PG-5% hydrogels demonstrating not significant difference to TCPS control during day 4 (Fig. 4 A). As compared with DPSCs, MG-63 osteosarcoma cells presented a decrease of metabolic activity from day 4 to day 7 in all samples, indicating a downregulated proliferation potential of osteosarcoma cells on WPI-PG hydrogels.”

5. Discussion

- The biocompatibility assessment should be discussed concerning ISO 10993-5 standard.

Response:

We thank the Reviewer for this comment and we added a sentence in the discussion.

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- [12] Y.P. Singh, S. Dasgupta, S. Nayar, R. Bhaskar, Optimization of electrospinning process & parameters for producing defect-free chitosan/polyethylene oxide nanofibers for bone tissue engineering, *Journal of Biomaterials Science, Polymer Edition* 31(6) (2020) 781-803.
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Highlights

- Whey Protein Isolate (WPI) hydrogels were enriched with different concentrations of phloroglucinol (PG).
- Incorporation of PG into WPI hydrogels increased the solubilization of PG.
- The addition of PG into the WPI hydrogels did not lead to any decrease of the mechanical properties.
- The WPI-PG hydrogels demonstrated antimicrobial activities against a range of bacteria causing common healthcare-associated infections.
- WPI-PG hydrogels supported the growth and collagen production of DPSCs for the formation of the extracellular matrix.
- WPI modification with PG supported mesenchymal stem cell adhesion and growth, inhibited cancer cell growth and adhesion.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Authors statement

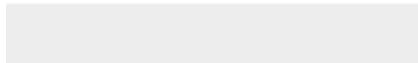
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Supplementary Material

Supplementary_submitted 10 08 2021.docx



Phloroglucinol-enhanced whey protein isolate hydrogels with antimicrobial activity for tissue engineering

Varvara Platania^{1,2}, Timothy E.L. Douglas^{3,4,*}, Mikhajlo K. Zubko⁵, Danny Ward⁶, Krzysztof Pietryga⁷, Maria Chatzinikolaidou^{1,2,*}

¹ Dept. of Materials Science and Technology, University of Crete, 70013 Heraklion, Greece

² Foundation for Research and Technology Hellas (FORTH)-Institute of Electronic Structure and Laser (IESL), 70013 Heraklion, Greece

³ Engineering Department, Lancaster University, UK

⁴ Materials Science Institute (MSI), Lancaster University, UK

⁵ Centre for Bioscience, Manchester Metropolitan University (MMU), Manchester, UK

⁶ Department of Biological and Life Sciences (BLS), Lancaster University, UK

⁷ Department of Biomaterials and Composites, AGH University of Science and Technology, Krakow, Poland

* Corresponding authors

Maria Chatzinikolaidou, mchatzin@materials.uoc.gr

Department of Materials Science and Technology, University of Crete, 70013 Heraklion, Greece

Tel. +30 2810394276

Timothy Douglas, t.douglas@lancaster.ac.uk

Engineering Department, Lancaster University, UK

ABSTRACT

Aging populations in developed countries will increase the demand for implantable materials to support tissue regeneration. Whey Protein Isolate (WPI), derived from dairy industry by-products, can be processed into hydrogels with the following desirable properties for applications in tissue engineering: (i) ability to support adhesion and growth of cells; (ii) ease of sterilization by autoclaving and (iii) ease of incorporation of poorly water-soluble drugs with antimicrobial activity, such as phloroglucinol (PG), the fundamental phenolic subunit of marine polyphenols.

In this study, WPI hydrogels were enriched with PG at concentrations between 0 and 20% w/v. PG solubilization in WPI hydrogels is far higher than in water. Enrichment with PG did not adversely affect mechanical properties, and endowed antimicrobial activity against a range of bacteria which occur in healthcare-associated infections (HAI). WPI-PG hydrogels supported the growth of, and collagen production by human dental pulp stem cells and - to a lesser extent - of osteosarcoma-derived MG-63 cells. In summary, enrichment of WPI with PG may be a promising strategy to prevent microbial contamination while still promoting stem cell attachment and growth.

Keywords: whey protein, phloroglucinol, hydrogels, osteogenic differentiation, cytocompatibility, dental pulp stem cells, MG-63 cells, antimicrobial activity

1. Introduction

Treatment of critical size bone defects generally requires surgeries involving implantation of autologous bone grafts or bone graft substitutes. Tissue engineering (TE) aims to develop innovative scaffolds for the regeneration of tissues. The approach of delivering stem cells to the defect site on scaffolds with osteoconductive properties to enhance bone healing has attracted great attention. The formation of new bone tissue depends on the osteogenic development of appropriate stem cells [1]. Besides the great efforts to treat large critical-sized bone defects, few clinical applications are available due to the limitations of materials and low functionality of the platforms [2] (REFS). Hydrogels possess great advantages in bone repair, as they are composed of three-dimensional [hydrophilic polymer](#) chains, which have superior mechanical strength and can provide nutrient environments suitable for endogenous cell growth. They are able to mimic the natural ECM, thus presenting a prospective ability to encapsulate bioactive molecules or cells. Additionally, raw materials for preparation of hydrogels are extensive and readily available, and they can be tailored to obtain the desired geometry for implantation or injection, and the degradation rate and porosity can be easily controlled by altering the crosslinking method and degree.

Recent advances in tissue engineering have focused on novel multifunctional biomaterials [3] based on renewable resources of great natural abundance, low cost and safety for human health. Protein-based eco-friendly biomaterials such as dairy proteins are currently of interest as substitutes for commercial materials for biomedical applications [4]. Whey proteins represent 20% of total milk proteins and are generally generated as a by-product of cheese manufacture. They are unique from a nutritional point of view as they contain all the essential amino acids, and they contain β -lactoglobulin, α -lactalbumin, immunoglobulins and bovine serum albumin [5, 6]. In addition, antimicrobial activity in milk is mainly attributed to some minor whey proteins such as lactoferrin, lysozyme, immunoglobulins, and lactoperoxidase [7, 8].

Furthermore, WPI solutions can form hydrogels upon heating; an important practical advantage of WPI hydrogels is that they withstand sterilization by autoclaving, a commonly used and clinically accepted sterilization technique. Hydrogels display several advantages when compared to other materials used for TE, including solid polymer scaffolds and ceramic scaffolds: they are non-brittle and incorporation of biologically active substances is straightforward.

Polyphenols have been implicated as components of antimicrobial hydrogels [9, 10]. Polyphenols are derived from plants and are known to possess antibacterial effects and antioxidative properties. This is beneficial in bone TE, as oxidative stress has been indicated to impede both the differentiation of osteoblasts and the resorption of bone by osteoclasts *in vitro* [11, 12]. The ability to ameliorate oxidative stress by phenolic compounds such as gallic acid isolated from terrestrial plants, and phloroglucinol (PG) found in seaweeds has been documented in numerous reports [13, 14]. PG has also been found to inhibit metastatic ability of breast cancer cells *in vitro* and *in vivo* [15]. The antibacterial effect of phlorotannins, polyphenols derived from brown seaweed of which PG is the basic subunit, is well-known [16], while recent research suggests that PG in its different forms also possesses antimicrobial activity [17, 18]. Healthcare-associated infections (HAI) are a burden worldwide, especially due to the increasing prevalence of antibiotic-resistant strains. Hence, antimicrobial activity is an added value for biomaterials used in TE applications.

Bone marrow (BM)-derived mesenchymal stem cells (MSCs) are the major cell source for bone TE. However, the morbidity of the donor site and painful procedures for harvesting these cells have limited their application [19]. Recently, human dental pulp stem cells (DPSCs) were introduced as an alternative to multipotent stem cells with self-renewal ability [20]. These cells could be harvested from both primary and permanent teeth during routine teeth extraction procedures in orthodontic treatment [20]. For bone TE, DPSCs showed a higher proliferative ability and number of colony clusters compared to BM-MSCs

[21]. Although other sources of MSCs have been applied for the regeneration of hard tissues, the complications at the site of harvest have limited their applications in practice. The DPSCs are an easily accessible source of MSCs with similar characteristics to bone marrow-derived stem cells and therefore can be used for hard tissue regeneration [22]. Due to their availability and differentiation potential, the application of these cells for TE has attracted enormous attention in recent years, including bone TE studies in animal models [21-24].

MG-63 cells derived from human osteosarcoma have been widely used in bone biology research to assess the *in vitro* osteogenic potential of biomaterials for bone TE and the pharmacokinetics for bone disease, as an alternative to normal osteoblastic cells due to ease of their availability and reproducibility of experimental results [25, 26]. MG-63 cells together with Saos2 and MC3T3-E1 cell lines are used as reliable and adequate cell culture models that resemble the behavior of primary osteoblasts. However, differences in phenotypic characteristics, proliferative capacity, secretion of osteogenic proteins and gene expression levels in MG-63 and osteoblasts have been reported, including the expression of abundant amounts of type III collagen and fibronectin in MG-63 cells [27]. The latter are constituents of soft connective tissue and absent in mineralized bone. Despite these differences, the MG-63 cell line is a useful cell model for *in vitro* biological assessment, including the evaluation of cell attachment, proliferation and osteogenic differentiation.

In this paper, DPSCs and MG-63 cells were cultured in the presence of WPI-PG hydrogels, namely WPI hydrogels modified with different concentrations of PG, and were evaluated for their viability, proliferation, cell adhesion, morphology, potential to enhance extracellular collagen production and antimicrobial properties. As this potential is expected to be different for cell lines and primary cells, physiological and cancer cell types, a comparative *in vitro* biocompatibility study was performed. Viability and proliferation of both cell types on the WPI hydrogels were quantified by means of the PrestoBlue® cell viability assay. Cell adhesion and morphology of both cell types on WPI-PG hydrogels were visualized by means of scanning electron microscopy (SEM). Moreover, the collagen levels in culture supernatants were determined as a marker of extracellular matrix (ECM) formation. Ten bacterial strains involved in healthcare associated infections (HAI), including Gram-positive and Gram-negative bacteria, were selected to perform sensitivity assays with WPI-PG hydrogels and evaluate their antimicrobial effects. All these strains present various types of primary and opportunistic HAI as well as food-borne and environmental infections. Seven of these strains belong to species included in the recently published list of drug-resistant bacteria that pose great health threats [28].

2. Materials and Methods

2.1 Hydrogel preparation

Stock solutions of 40% WPI (w/v) were prepared by dissolving 20 g of WPI powder in 50 ml of deionised H₂O. A sonicator was used to facilitate dissolution and minimize foaming. Subsequently, 15 ml of WPI stock solution was transferred to Falcon tubes, and the required amount of phloroglucinol (Sigma-Aldrich, Germany) was added. The WPI solutions enriched with 0 (control), 2.5, 5, 10 and 20% w/v PG concentrations are designated as WPI-PG-0%, WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10%, and WPI-PG-20%, respectively (Table 1). The WPI-PG solutions were vortexed until mixed homogeneously, and 15 ml were poured into 9 cm (in diameter) Petri dishes and autoclaved at 121°C for 15 minutes to simultaneously sterilise and induce gelation. Samples were then cooled to 60°C and cooled further at ambient temperature. The WPI-PG hydrogels were cut into disc-shaped samples by means of a biopsy punch with a diameter of 8 mm and a weight of approximately 50 mg.

Table 1. WPI hydrogels enhanced with different concentrations of PG. The WPI content of 40% (w/v) was the same for all WPI-PG hydrogels.

Material designation	PG content (%w/v)
WPI-PG-0% (control)	0
WPI-PG-2.5%	2.5
WPI-PG-5%	5
WPI-PG-10%	10
WPI-PG-20%	20

2.2 Surface topography studies

Sample cross-sections comprising a range of WPI-PG hydrogels were viewed by SEM. SEM analysis was performed using a 7800F JEOL SEM instrument. Prior to SEM analysis, samples were mounted on aluminum stubs (Agar Scientific Ltd, UK) using carbon adhesive pads (Agar Scientific Ltd, UK) and coated with a 10 nm layer of gold using a Q150RES sputter coater (Quorum Technologies Ltd). SEM was used to analyze differences in the surface topography of WPI-hydrogels modified with PG concentrations ranging from 0 to 20%.

2.3 Mechanical testing

The effects of PG incorporation into WPI hydrogels on the hydrogels' mechanical properties were evaluated. Cylindrical WPI samples of 2.5, 5, 10 and 20% PG, measuring 10 mm in height and 8 mm in diameter, were subject to compressive testing using a Zwick 1435 Universal Testing Machine. Test parameters were set at a speed of 10 mm per minute, and a test range of 0-95% strain.

2.4 DPSCs and MG-63 cell culture maintenance

DPSCs cultures were established after generous donation by Prof. A. Bakopoulou at the School of Dentistry at the Aristotle University of Thessaloniki, Greece. Based on a previously described protocol [22], DPSCs cultures were established using an enzymatic dissociation method following their isolation from wisdom teeth of healthy donors after informed consent according to the Declaration of Helsinki (ethical approval number:

322/15-4-2013 by the Institutional Ethics Committee). Briefly, following disinfection of third molars, a cut was made around the cementum-enamel junction to expose the pulp chamber. Then, the pulp was retrieved, thoroughly minced and digested for 45 min at 37°C with a mixture of 3 mg/ml of collagenase I and 4 mg/ml of dispase II (Invitrogen, Karlsruhe, Germany). DPSCs were cultured in minimum essential media α -MEM (Invitrogen) supplemented with 15% fetal bovine serum (FBS, Invitrogen), 100 mM L-ascorbic acid phosphate (Sigma-Aldrich, Steinheim, Germany), 100 units/ml of penicillin, 100 mg/ml of streptomycin and 1% amphotericin as previously described by the same team [22]. All experiments were conducted with DPSCs at passages 2 to 4. The osteosarcoma cell line MG-63 was purchased from Sigma (USA) and cultivated in DMEM containing 100 units/ml of penicillin, 100 mg/ml of streptomycin and 1% amphotericin (all from Gibco), supplemented with 10% fetal bovine serum (FBS) according to bibliography [29-31]. Cells were maintained in a humidified atmosphere under 5% CO₂ at 37°C, and the media were changed twice a week.

2.5 Cell viability and proliferation assay

The viability and proliferation of DPSCs and MG-63 on WPI-PG hydrogels was quantitatively assessed using the resazurin-based metabolic assay PrestoBlue® (Invitrogen, USA) as previously described [32]. Briefly, the disc-shaped WPI-PG scaffolds, generated by using an 8 mm biopsy cutter, were placed into 48-well plates and each sample was seeded with 2×10^4 cells. At each experimental time point of 2, 4 and 7 days of culture, the PrestoBlue® reagent was added directly to the wells at a 1:10 ratio in culture medium and incubated at 37°C for 60 min, before measuring the absorbance at 570 and 600 nm in a spectrophotometer (Synergy HTX Multi-Mode Microplate Reader, BioTek, Winooski, VT, USA). Three independent experiments were performed in triplicate.

2.6 Adhesion and morphology of the DPSCs and MG-63 cells on the WPI-PG hydrogels

The adhesion and morphology of DPSCs and MG-63 osteoblast-like cells were observed using SEM (JEOL JSM-6390 LV) after 2, 4 and 7 days in culture. Seeded scaffolds with 2×10^4 cells per sample were placed in a CO₂ incubator at 37°C for 2, 4 and 7 days and then were removed from the incubator and rinsed three times with PBS, fixed with 4% v/v para-formaldehyde for 20 min and dehydrated in increasing concentrations (30–100% v/v) of ethanol. The scaffolds were then dried in a critical point drier (Baltec CPD 030), sputter-coated with a 20 nm thick layer of gold (Baltec SCD 050) and observed under SEM at an accelerating voltage of 15 kV.

2.7 Determination of the produced extracellular collagen

A modified Sirius red assay [33] was used to stain total collagen produced in the extracellular matrix. The anionic dye Sirius Red binds to the [Gly–X–Y] tripeptide of mammalian cells. Collagen is a basic protein, and it is thus likely that the sulphonic groups of the dye may interact at low pH with the amino groups of lysine and hydroxylysine, and with the guanidine groups of arginine [34]. On days 2, 4 and 7 of the experiment, the culture supernatants were collected, and 25 μ l were diluted to a final volume of 100 μ l with ultrapure water. Then 1 ml of the dye solution consisting of 0.1% w/v Sirius red F3B (Sigma-Aldrich, St. Louis, MO, USA) in 0.5 M acetic acid was added, and the samples were incubated at room temperature for 30 min. The samples were centrifuged at 15,000 g for 20 min to pellet the collagen–dye complex. The pellet was washed with 0.5 ml of 0.5 M acetic acid to remove the unbound dye. Dissolution of the collagen–dye complex with 1

ml of 0.5 M NaOH followed, and 200 µl of the solution were transferred into 96-well plates to measure the absorbance at 530 nm (Synergy HTX Multi-Mode Microplate Reader, BioTek, Winooski, VT, USA). Collagen concentration was determined by means of a calibration curve. Samples were analyzed in quadruplicate.

2.8 Microbiological assays

Bacterial strains used in the study included *Bacillus cereus* (MCIMB 9373), *Enterococcus faecalis* (NCTC 12697), *Escherichia coli* (NCTC 9001), *Klebsiella oxytoca* (ATCC 15764), *K. pneumoniae* (40602), *Pseudomonas aeruginosa* (NCTC 6749), *Staphylococcus aureus* (NCTC 6571), *S. aureus* (MRSA) [originated from NCTC 13552], *S. epidermidis* (NCTC 11047), *Streptococcus pyogenes* (NCIMB 8198).

For susceptibility testing, the bacterial strains were grown overnight on nutrient agar (Oxoid CM0003) at 37°C. Inocula were prepared according to the standard protocol (Andrews and BSAC Working Party On Susceptibility Testing ft 2001). Disc-shaped WPI-PG hydrogels were prepared and placed onto a bacterial inoculum. For sensitivity tests, the samples were placed on the surface of plates containing 25 ml of Iso-sensitest agar (Oxoid CM0471) or (in some experiments) nutrient agar. Before placing the samples, the agar plates were swabbed with microbial suspensions. The plates were incubated overnight at 37°C. Zones of inhibition (ZOI) were recorded after 18-30 h when bacterial lawns became clearly visible on the agar.

To test modes of inhibition of bacterial growth, the samples of hydrogels were removed from ZOI using sterile forceps, and central areas of ZOI without any signs of bacterial growth were touched firmly a few times with sterile wet swabs, streaked out (in single lines) onto sectors on nutrient agar plates (Supplementary Fig. 2), and incubated overnight at 37°C. The absence of bacterial growth on the streaked areas was interpreted as bactericidal inhibition; the restoration of growth indicated bacteriostatic effects.

2.9 Statistical analysis

The experimental data were analyzed using two-way ANOVA followed by Tukey's multiple comparisons test among groups. Data were expressed as means ± standard deviation (SD). For these analyses the GraphPad Prism software version 8.0 (La Jolla, USA) was used. The symbols * designate $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns denotes statistically non-significant differences.

3. Results

3.1 WPI-PG hydrogels and surface characterization by means of SEM

Sterile, disc-shaped WPI-PG hydrogels were prepared as described in section 2.1, and based on their PG concentration they were denoted as WPI-PG-0% (control), WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10%, and WPI-PG-20%. The WPI-PG hydrogels changed colour from yellowish for WPI-PG-0% to dark brown for the WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10%, and WPI-PG-20% (Fig. 1).

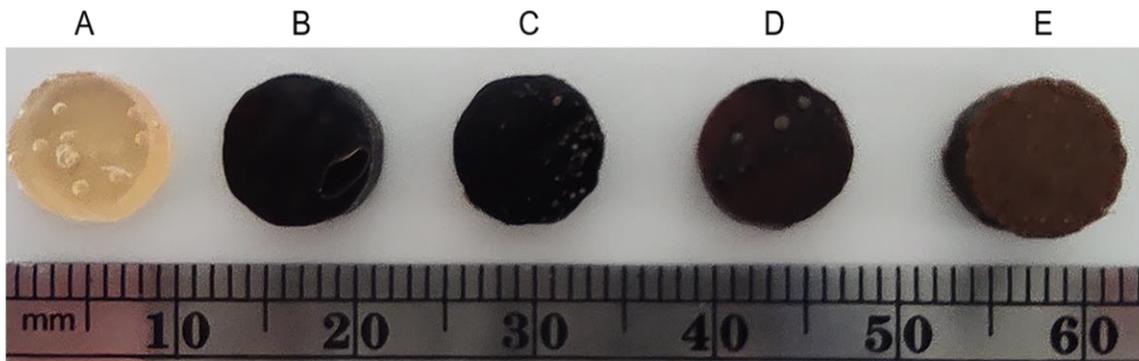


Fig. 1: Macroscopic images of disc-shaped samples prepared from hydrogels solidified and autoclaved in petri dishes. A: WPI-PG-0%, B: WPI-PG-2.5%, C: WPI-PG-5%, D: WPI-PG-10%, E: WPI-PG-20%.

The microstructures of WPI-PG hydrogels with concentrations ranging from 0 to 20% PG were analyzed via SEM (Fig. 2). The WPI control (0% PG) sample presented a consistently smooth surface topography. It is conceivable that the increase in roughness is caused by the presence of PG in the hydrogel, which may form deposits when hydrogels are dried during preparation for SEM studies.

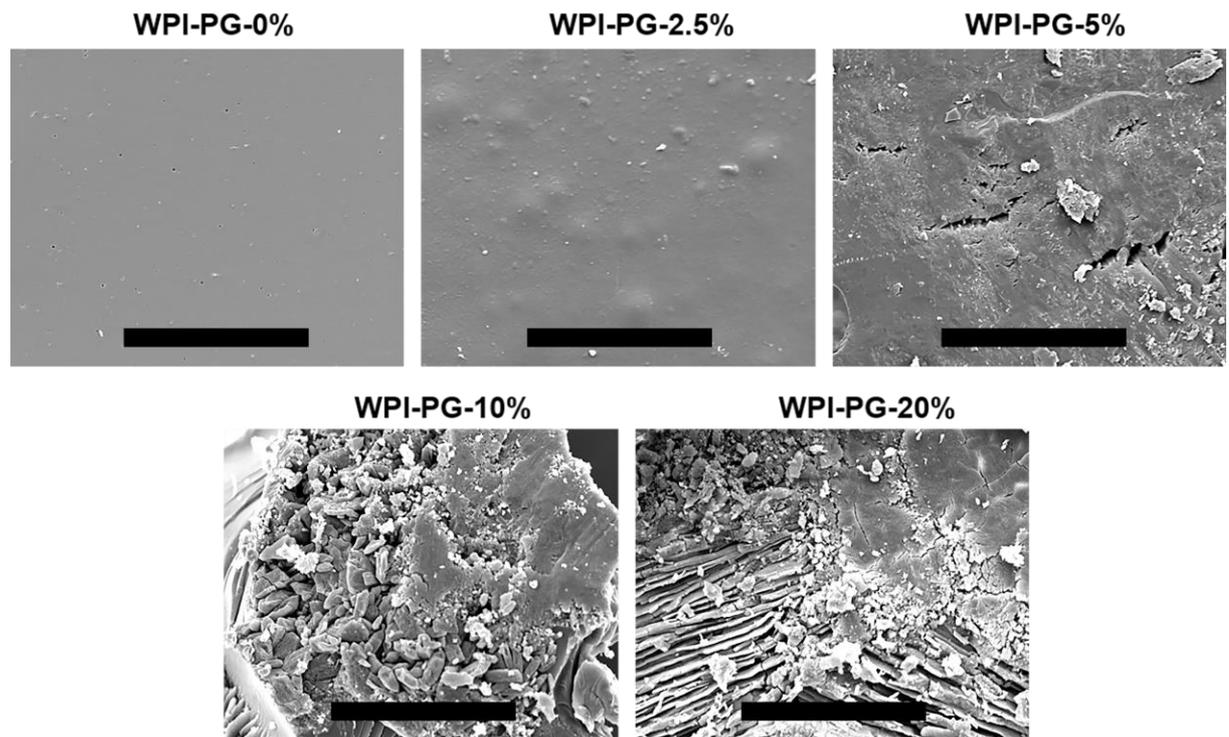


Fig. 2. SEM images displaying alterations in surface topography of WPI hydrogels containing PG at concentrations of 0%, 2.5%, 5%, 10% and 20%. Scale bars on SEM images indicate 1000 μm .

3.2 Mechanical testing

The WPI-PG-0%, WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10% and WPI-PG-20% hydrogels were characterized for mechanical properties by measuring their compressive strength,

Young's modulus and % strain at break (Fig. 3). It is important to note that, due to high friction caused by the nature of the samples, the WPI hydrogels supplemented with 20% PG became stuck in the jaws of the Zwick testing machine, meaning values for strength, modulus and strain at break may have been overestimated.

3.2.1 *Compressive strength*

The compressive strength values for WPI hydrogels containing 0, 2.5, 5, 10 and 20% PG are shown in Fig. 3A. No statistically significant difference in compressive strength was observed between the WPI control (0% PG) and the 2.5% PG sample group (mean values of 4.26 and 4.34 MPa, respectively). However, increasing the PG concentration to 5% resulted in the compressive strength decreasing by 1.35 MPa to a mean of 2.91 MPa. This reduction was deemed to be statistically insignificant compared to both the WPI control and the 2.5% PG sample group ($P < 0.05$). Increasing the PG concentration to 10% led to a mean compressive strength of 5.06 MPa. However, values were inconsistent within this group and no statistical significance was proven. Values for the 20% PG sample group were significantly higher by a factor of approximately three compared to the other groups; however, these values may have been overestimated.

3.2.2 *Young's modulus*

Except for the 20% PG sample group for which values may have been overestimated, no statistically significant difference in Young's modulus was observed between PG supplemented sample groups compared to the WPI control (Fig. 3B). The addition of 2.5% PG resulted in a negligible increase in Young's modulus, while 5% PG caused a negligible decrease. A statistically significant increase of 0.43 MPa was observed when PG concentration was increased from 5% to 10%. However, all samples demonstrated a Young's modulus between 3 and 4 MPa.

3.2.3 *Strain at break*

The % strain at break of the WPI samples with different PG concentrations is presented in Fig. 3C. The mean deformation observed for the WPI control (0% PG) sample group was 7.09 mm (70.92%). The presence of 2.5% PG in WPI hydrogels resulted in a slight increase in the strain at break (72.31%) while supplementation of 10% decreased this value to 70.14%. However, except for the potential overestimate in the 20% PG samples, the only statistically significant difference ($p < 0.05$) observed for strain at break coincided with the addition of 5% PG (61.78%).

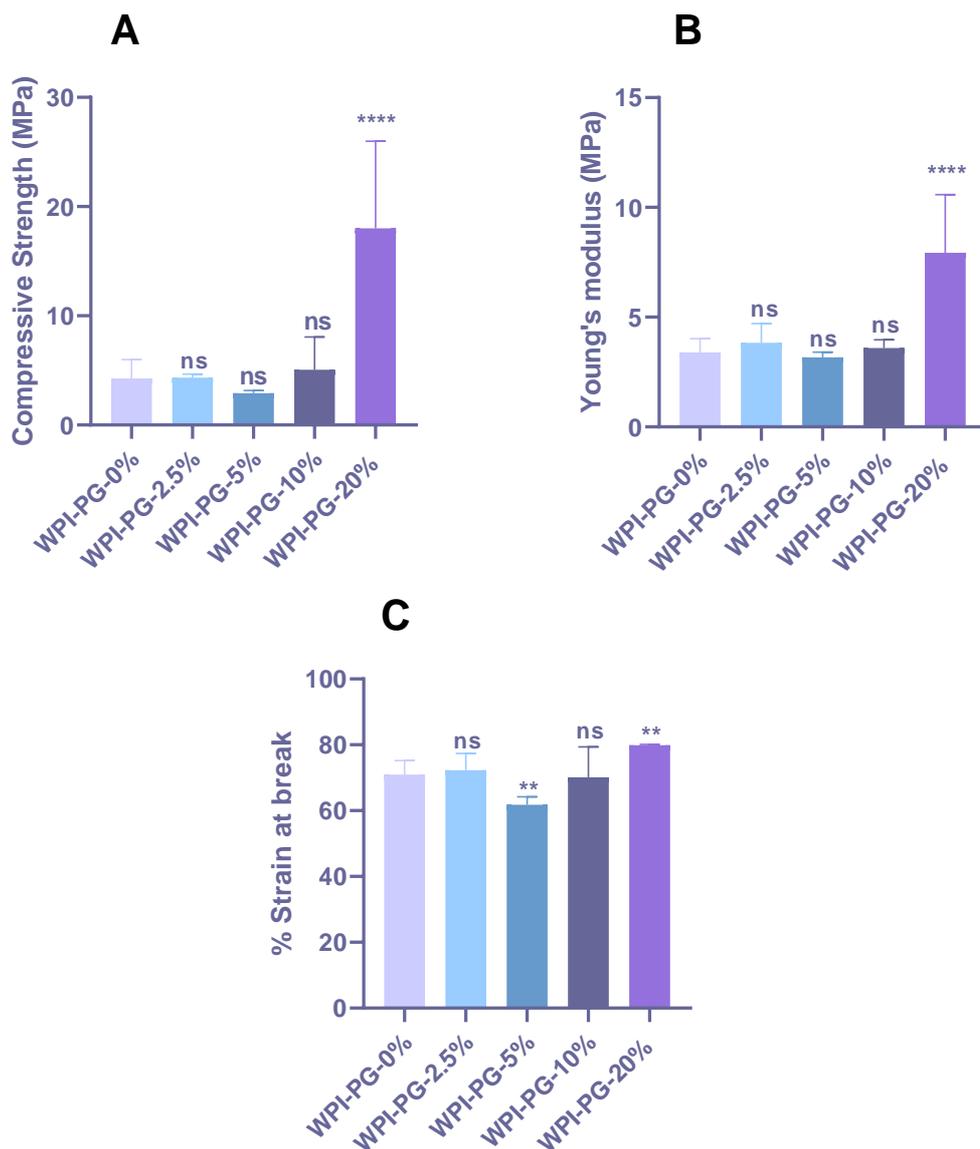


Fig. 3. Mechanical testing of WPI hydrogels containing 0, 2.5, 5, 10 and 20% PG. A: Compressive strength; B: Young's modulus; C: Strain at break. Each bar represents the mean \pm SD of nine replicates (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; ns denotes statistically non-significant difference compared to the WPI-PG-0 control).

3.3 Cell viability and proliferation

Cellular viability and proliferation of DPSCs and MG-63 on WPI-PG-0%, WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10% and WPI-PG-20% hydrogels were quantitatively assessed by measuring their metabolic activity after 2, 4 and 7 days in culture (Fig. 4). For the whole incubation period, cell numbers for both cell types investigated were found to be higher on the tissue culture treated polystyrene (TCPS) control than on the WPI-PG hydrogels (Fig. 4 A, C).

DPSCs displayed increased metabolic activity, expressed as absorbance value, from day 4 to day 7 in all samples, with the WPI-PG-10% and WPI-PG-5% hydrogels demonstrating non-significant difference to TCPS control during day 4 (Fig. 4 A). As compared with DPSCs, MG-63 osteosarcoma cells presented a decrease of metabolic activity from day 4

to day 7 in all samples, indicating a downregulated proliferation potential of osteosarcoma cells on WPI-PG hydrogels.

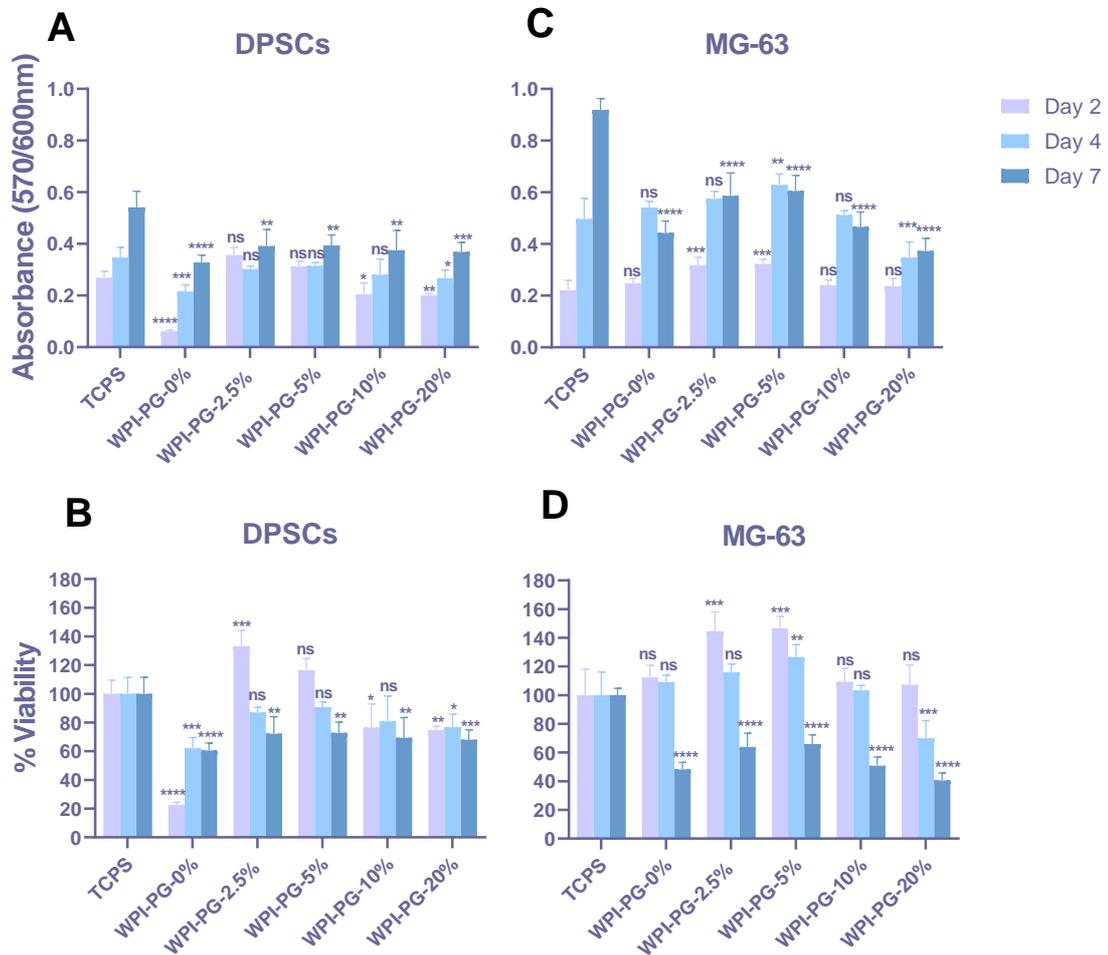


Fig. 4. Cell viability and proliferation of DPSCs (A, B) and MG-63 (C, D) cells cultured in the presence of WPI hydrogels with different PG concentrations, expressed as absorbance (A, C) and % viability of the TCPS control set at 100% for each experimental time point (B, D). Each bar represents the mean \pm SD of quadruplicates (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns denotes a statistically non-significant difference compared to the TCPS control at the corresponding time point).

3.4 Effects of WPI-PG hydrogels on cell adhesion, morphology and growth

Representative SEM images show DPSCs on WPI-PG-0%, WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10% and WPI-PG-20% hydrogels, 4 and 7 days post-seeding (Fig. 5). Images at days 4 and 7 showed cell spreading for increased PG concentrations in WPI-PG-10% and WPI-PG-20% hydrogels with DPSCs, however without displaying their characteristic spindle shape morphology until day 7. On day 7 in culture, DPSCs displayed cell-cell interactions that promote tissue formation. Cell infiltration within the pores of the WPI-PG-

10% and WPI-PG-20% hydrogels was observed in Fig. 5. In contrast, MG-63 cells appear round on the surface of the hydrogels with an obvious decrease in cell density on WPI-PG-2.5% and WPI-PG-5% hydrogels (Fig. 6). On the latter two compositions the observed cell density of DPSCs was lower as well.

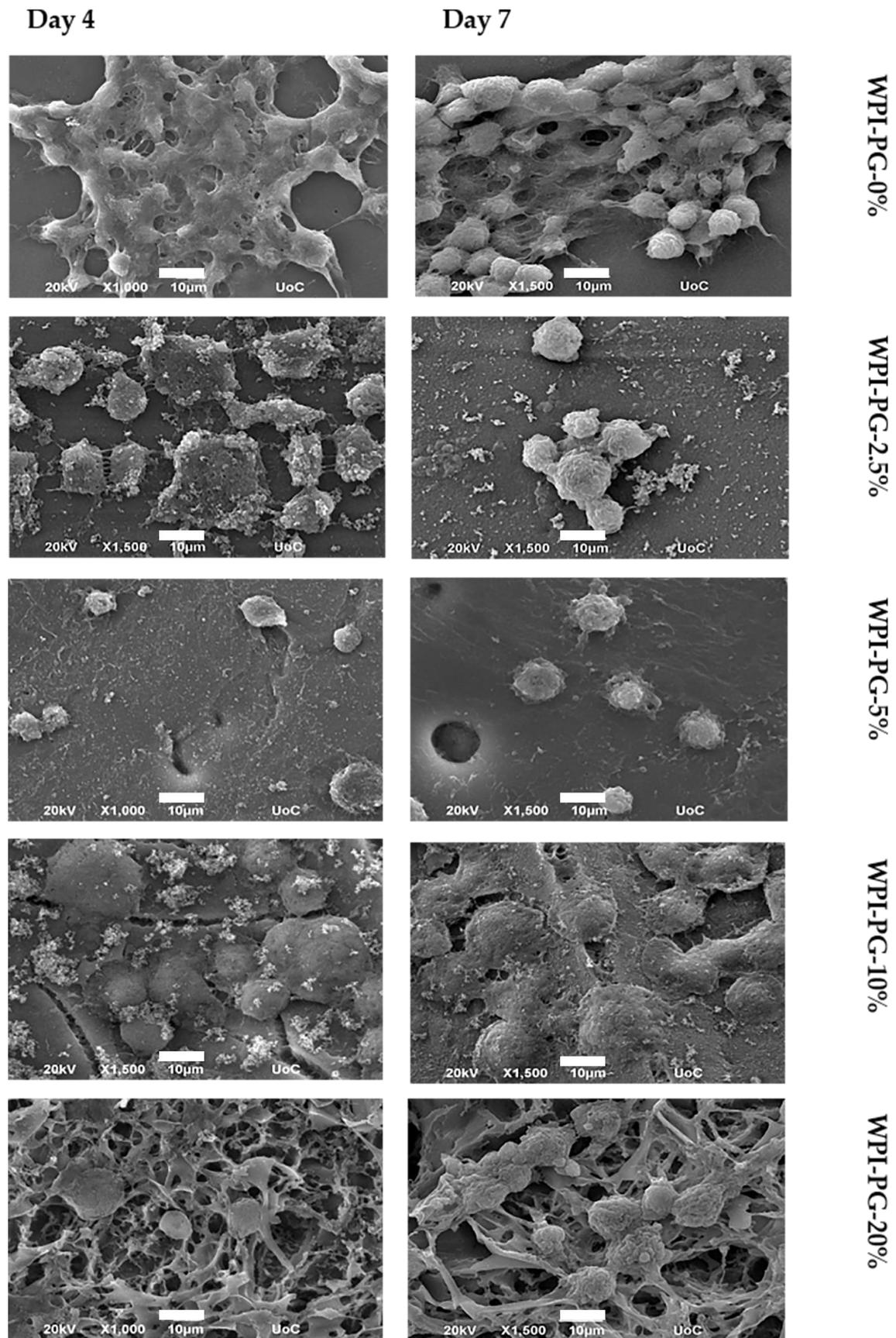


Fig. 5. Evaluation of cell attachment and morphology of DPSCs cultured on WPI hydrogels modified with different PG concentrations after 4 and 7 days in culture. Scale bars represent 10 μ m.

The morphology of MG-63 cells on WPI-PG-0%, WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10% and WPI-PG-20% hydrogels on day 4 and 7 was visualized via SEM and presented in Fig. 6. WPI-PG-10% and WPI-PG-20% hydrogels promoted cell spreading.

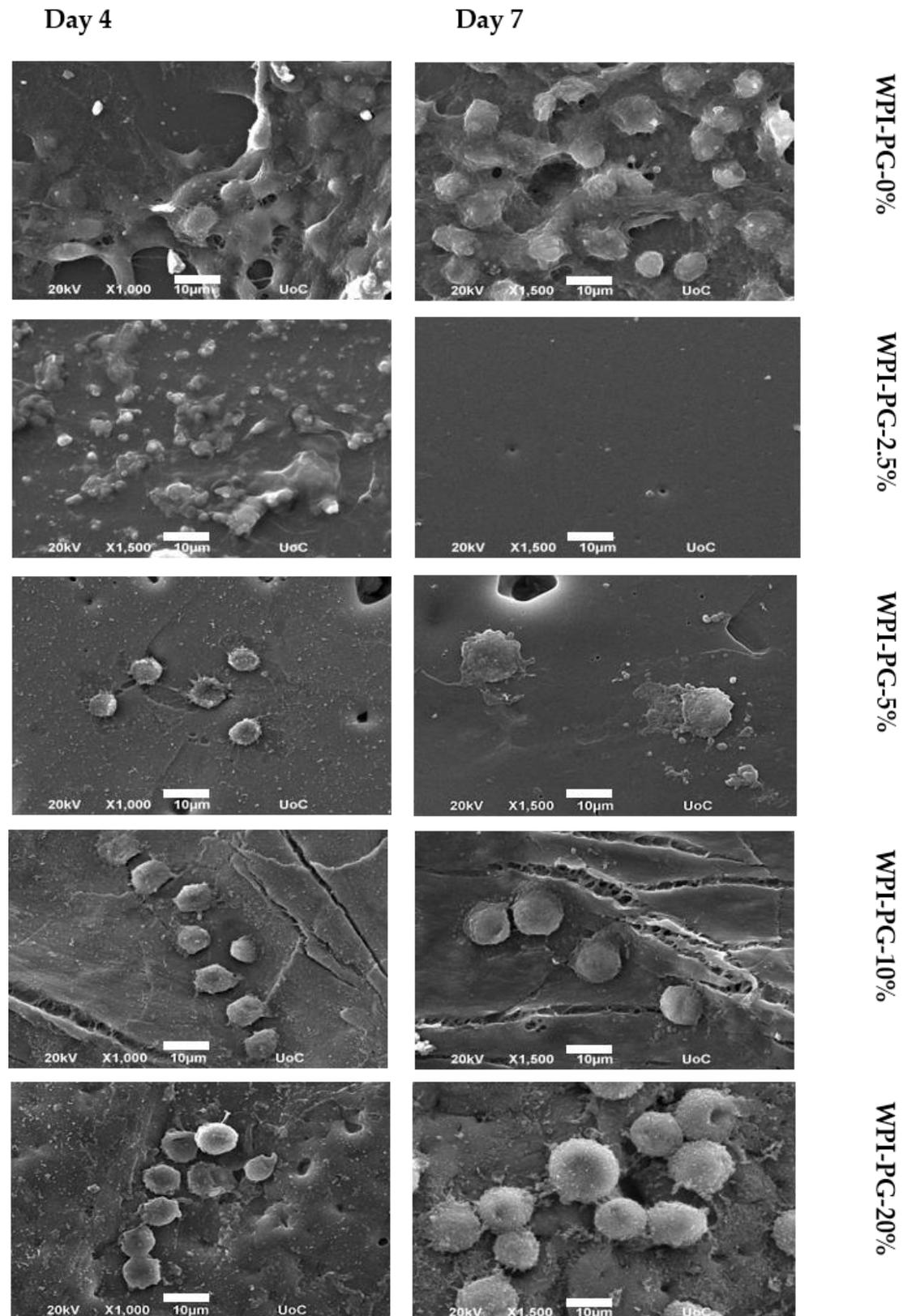


Fig. 6. Evaluation of cell attachment and morphology of MG-63 osteosarcoma cells cultured on WPI hydrogels modified with different PG concentrations, after 4 and 7 days in culture. Scale bars represent 10 μm .

3.5 Extracellular collagen production

In order to investigate the potential of ECM formation by DPSCs and MG-63 cultured on the WPI-PG hydrogels, the secretion of collagen was determined, as collagen constitutes the main structural element of the ECM. We quantified the total collagen secreted in culture supernatants at different time intervals, as shown in Fig. 7, in the presence of exogenous ascorbic acid which is a co-factor in collagen synthesis [35]. We found that DPSCs produced similar levels of collagen on days 2 and 4 in the presence of all WPI-PG hydrogels. On day 7, significantly lower values were observed for WPI-PG-2.5%, WPI-PG-5%, and WPI-PG-10% compared to the WPI-PG-0% control, while for WPI-PG-20% they were the highest among the hydrogels and similar to the control. MG-63 osteosarcoma cells exhibit significantly lower levels of collagen production compared to the DPSCs cultures. Collagen production levels in MG-63 cultures were similar to the WPI-PG-0% control on days 2 and 4, except of the WPI-PG-20% hydrogels that indicated significantly higher values than the control on day 2.

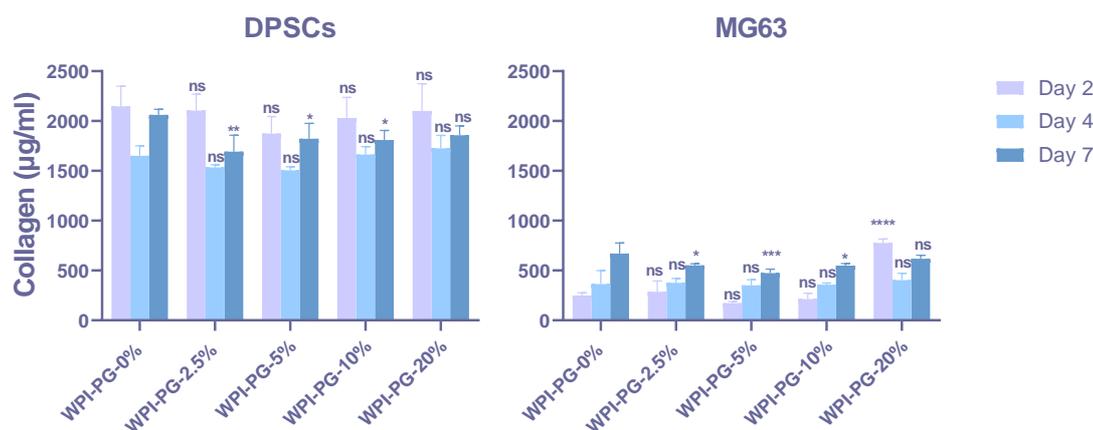


Fig. 7. Levels of total collagen in the supernatants of the DPSCs (left) and the MG-63 cells (right) cultured on the different PG concentrations in WPI-hydrogels for 2, 4 and 7 days. Error bars represent the average of quadruplicates (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns denotes statistically non-significant difference compared to the WPI-PG-0% control at the corresponding time point).

3.6 Antibacterial properties of WPI-PG hydrogels

First assays involving testing of antimicrobial properties of disc-shaped WPI-PG hydrogels were carried out against a MRSA strain. The examples of these experiments are shown in Supplementary Fig. 1. It is evident from Supplementary Fig. 1a that 5 mg of the WPI-PG-2.5%, WPI-PG-5% and WPI-PG-10% hydrogels did not inhibit the MRSA growth, but there was slight growth inhibition with a narrow ZOI in WPI-PG-20%. The assay involving 20 mg

of WPI-PG-20% showed a clear ZOI. To enhance the assay sensitivity, the amount of the hydrogels was increased to 30 mg in the next experiment (Supplementary Fig. 1b), in which five bacterial species (*Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Escherichia coli* and *Streptococcus pyogenes*) manifested clear ZOI, and one species (*Enterococcus faecalis*) was marginally inhibited in its growth. These results suggest an obvious antibacterial potential of the hydrogel at least against seven bacterial species. Moreover, disc-shaped hydrogel samples were used for the sensitivity testing, and the results are shown in Fig. 8. All four bacterial species used in this experiment were clearly inhibited in their growth by WPI-PG hydrogels at all used concentrations of PG on two different media (Iso-sensitest and nutrient agar). The level of inhibition (ZOI size) was dependent on PG concentrations. Overall, the inhibitory effects were stronger and clearer on Iso-sensitest agar than on nutrient agar, although the results for *P. aeruginosa* were comparable on both media. The blank WPI hydrogels without PG did not manifest any inhibitory effects.

After recording the results on sensitivity testing (next day following swabbing), we reused the plates with ZOI for determining the mode of growth inhibition in a simple assay (see Methods). Supplementary Fig. 2 shows an example of results from such assay performed for WPI-PG-10% and WPI-PG-20% hydrogels within the experiment depicted in Fig. 8. The mode of inhibition depended on PG concentrations and media used. Bactericidal growth inhibition was observed for WPI-PG-20% hydrogels in *P. aeruginosa* on both media, and in *S. epidermidis* on Iso-sensitest, as well as for WPI-PG-10% hydrogels in *S. epidermidis* on Iso-sensitest. Predominantly bactericidal inhibition with a bacteriostatic effect for a low proportion of cells was observed for WPI-PG-20% hydrogels in MRSA on both media, and in *K. oxytoca* on nutrient agar. WPI-PG-10% hydrogels showed bacteriostatic inhibition on both media for MRSA, *K. oxytoca* and *P. aeruginosa*. Bacteriostatic inhibition was also observed for WPI-PG-20% hydrogels in *K. oxytoca* on Iso-sensitest, and in *S. epidermidis* on nutrient agar.

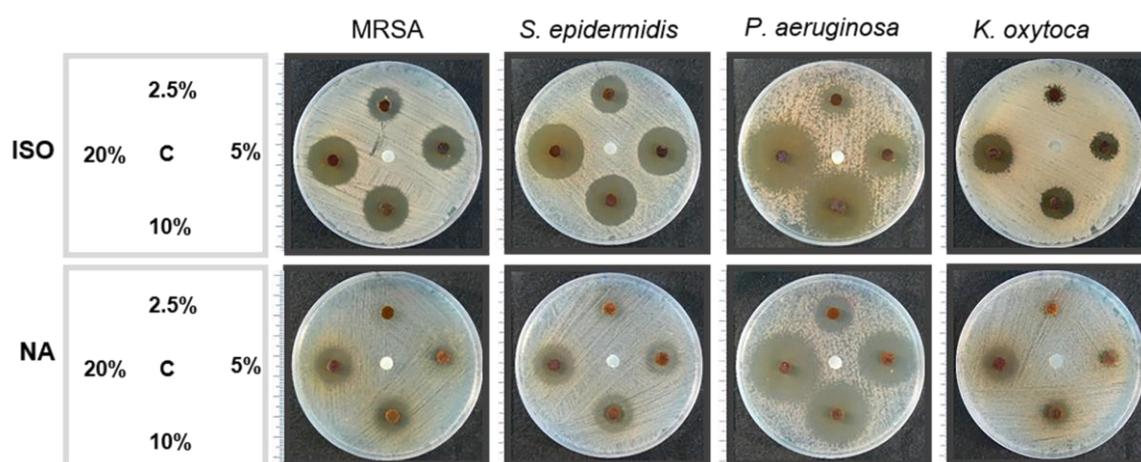


Fig. 8. Inhibition of bacterial growth by WPI-PG hydrogels on Iso-sensitest agar (ISO) and nutrient agar (NA). The squares on the left show the arrangement of hydrogel discs with different concentrations of PG and without PG (C). The ruler (with 1 mm scale) is attached to the left side of each image. The diameter of each plate is 9 cm.

4. Discussion

Recent advances in TE focus on finding more biocompatible, biodegradable and non-toxic biomaterials with the ability to mimic the natural physiological environment and favor enhanced cell-material interactions. Protein-based biomaterials have the characteristics of improved bioresorbability, biocompatibility, tissue healing, and ability to promote regeneration [4, 36-38]. In this work we report on WPI and PG for the development of multifunctional biomaterials for biomedical applications. PG was identified as a component of hydrogels due to its known antimicrobial and antioxidative properties. The investigation of biomaterials suitability for bone TE includes their capacity to promote cell proliferation, adhesion and ECM formation. We demonstrated that DPSCs and MG-63 showed different cellular responses when seeded on different WPI-PG hydrogels, which is in agreement with literature reports [25].

The microstructure of WPI hydrogels has been examined by means of SEM. As expected, the control WPI hydrogels (0% PG) displayed a homogeneously smooth surface topography in the dry state [6]. With increasing PG concentration, hydrogels were observably rougher in the dry state. This may be an advantage for their potential applications in bone TE, as both stiffer [39] and rougher [40] surfaces are known to promote the differentiation of cells towards the osteoblastic phenotype.

Minimal increases in both Young's modulus and percentage strain at break were found in hydrogels supplemented with PG at lower concentrations of 2.5%. Since stiffer surfaces are known to promote the differentiation of cells towards the osteoblastic phenotype, PG imparted stiffness would improve the compatibility of these hydrogels with bone tissue. However, this effect was not observed in a dose-dependent manner. The values for Young's modulus, compressive strength and percentage strain at break remained relatively consistent throughout sample groups, even when the concentration of PG was increased to 10%. This suggests that the incorporation of PG into the WPI hydrogels has no significant impact on the overall structural function of WPI hydrogels up to a PG concentration of 10%. Values for hydrogels containing 20% PG were higher; however, it is important to note that, due to high friction, the WPI hydrogels supplemented with 20% PG remained stuck in the jaws of the testing machine, and therefore it cannot be concluded that this PG concentration improves the aforementioned mechanical properties. As such, PG supplementation at higher concentration may alter the mechanical properties of WPI composites, resulting in 'stickier' hydrogels. In future work, one might consider strategies to reduce adhesion between samples and the testing machine jaws with minimal influence on mechanical properties, for example the insertion of a thin, non-adhesive piece of material between the jaws and the sample.

Discussion of the mechanisms for the effect (or lack of effect) of PG on WPI hydrogels' mechanical properties must remain speculative in view of the limited data available. It is known that phlorotannins interact with proteins, resulting in a dark brown colour [41], which was ascribed to non-covalent protein-phlorotannin interactions. One may expect similar interactions between proteins and PG, the fundamental subunit of phlorotannins, which would explain the brown color observed in this study (Fig. 1). Possibly, the number of crosslinks caused by interactions between WPI proteins and PG must exceed a certain number in order to markedly influence mechanical properties. The increased "stickiness" of hydrogels containing 20% PG might be due to interactions between PG bound to the WPI hydrogel network and surfaces; hydrogels containing polyphenol groups have exhibited increased adhesiveness [42]. Again, one can speculate that a certain PG concentration should be exceeded before such "stickiness" becomes appreciable. Alternatively, it is possible that at high PG concentrations, some PG may have "precipitated" as particles within the hydrogels after cooling, which might conceivably cause mechanical reinforcement. However, as already mentioned, such discussion must remain speculative.

Since materials for biomedical applications are potential sites for exogenous and endogenous infections, it is beneficial if they possess antimicrobial properties [43]. To evaluate them, we used a simple and convenient approach of making disc-shaped hydrogel samples allowing easy standardization of weight, volume, shape and application

of hydrogel materials to microbial inocula. These advantages of disc-shaped samples make them comparable in efficiency to paper discs used routinely in sensitivity assays involving liquids [44, 45]. Using these samples allows quantification of sensitivity data (Supplementary Fig. 3).

Overall, all ten Gram-positive and Gram-negative bacteria used in our study were inhibited (in bactericidal or bacteriostatic mode) by WPI-PG hydrogels, suggesting presumably non-specific inhibitory mechanisms. Interestingly, *Candida albicans* was not inhibited by WPI-PG hydrogels (as shown in Supplementary Fig. 4), suggesting that eukaryotic cells might be generally tolerant to PG, which is consistent with our results for human cells cultivated *in vitro*. Modes of inhibition were determined by using a simple “streak out assay” to check re-growth of inhibited bacterial cells taken directly from ZOI. This assay is convenient and rapid. Although WPI itself possesses antimicrobial properties [46], we never observed inhibition of bacterial growth by WPI hydrogels without PG, probably due to the denaturation of proteins during hydrogels formation.

Ideally, an antibacterial biomaterial would exhibit both a burst release to prevent microbial colonization and help the recipient's cells win the “race to the surface”, and also a prolonged release to prevent later microbial colonization. For this, more detailed studies of PG release are necessary in future work. In addition, testing of antimicrobial properties of samples after incubation in biological fluids for a more extended period of time would be prudent to verify the mid- to long-term antimicrobial activity.

Regarding the cell viability assessment of the DPSCs and MG-63 cells on the WPI-PG hydrogels, we quantitatively evaluated the cytotoxic effect of substrates according to ISO 10993-5, in which it is stated that reduction of cell viability by more than 30% is considered a cytotoxic effect. In accordance, no cytotoxic effect was observed on days 2 and 4 for both cell types that displayed viability rates from 87-145% compared to the TCPS control, with higher values on WPI-PG-2.5% and WPI-PG-5% hydrogels on day 2. On day 7, the cell viability for DPSCs ranged from 60% to 73% compared to the control, while MG-63 cells presented viability values from 40% to 65%. Increased PG concentrations led to an inhibition of the MG-63 proliferation, consistent with the previously reported anti-cancer activity of PG [13, 15].

Collagen is the most abundant fibrous protein within the interstitial ECM that constitutes up to 30% of the total protein mass in mammals, providing tensile strength, regulating cell adhesion, supporting chemotaxis and migration, and directing tissue development [47]. Deposition of type I collagen-rich ECM is a prerequisite for the expression of specific osteoblast products, such as alkaline phosphatase, during the normal developmental sequence of the osteoblast [48]. Collagen levels secreted by DPSCs cultured on WPI-PG hydrogels are increased two to three-fold compared to the MG-63 secretion levels. These results suggest that WPI-PG hydrogels may have supported the ECM formation when seeded with DPSCs.

SEM micrographs of the 2D WPI-PG hydrogel surface underline the relationship between the increasing concentration of PG in the hydrogel and the supported adhesion and proliferation of DPSCs and MG-63 cells. In addition to cell adhesion, the morphology of the cells was evaluated. MG-63 osteosarcoma cells exhibited a round shape morphology in contrast to DPSCs, which were found to form cell-cell and cell-material interactions after 4 and 7 days in culture. Although MG-63 cells are widely used for biochemical studies of osteoblast-specific functions *in vitro*, their abundant type III collagen synthesis has been known to be unusual compared to other osteoblast lineages [49]. MG-63 osteosarcoma cells have been characterized as an atypical bone-forming model since the gene expression profile and the capability to synthesize a correct extracellular bone matrix are impaired [50]. It is also possible that the tumorigenic nature of MG-63 prevents them from properly adhering onto the WPI-PG hydrogels. In addition, the DPSCs culture contained ascorbic acid, a required cofactor for enzymes to hydroxylate proline and lysine in pro-collagen, enabling the collagen chains to form a proper helical structure [51]. The role of ascorbic acid in osteogenic differentiation is mainly attributed to the secretion of type I collagen into the ECM. The significantly higher total collagen production found in DPSCs

cultures on WPI-PG hydrogels compared to the MG-63 cells is in line with the cell-cell interactions observed for DPSCs by means of SEM indicating good adhesion and a high affinity to the substrate, similar to a previous study displaying strong attachment of DPSCs onto chitosan/gelatin scaffolds that enhanced bone tissue formation *in vitro* and *in vivo* [22]. The presence of cell-cell interactions suggests active cell motility, which is essential for tissue formation within a scaffold, and this is in agreement with a recent report on dense ECM formation by DPSCs cultured onto biopolymer-based hydrogels that promoted the osteogenic response *in vitro* [52]. Another study towards bone TE demonstrated the capacity of DPSCs to strongly attach on antibacterial thymol-functionalized hybrid scaffolds, increasing proliferation and calcium mineralization [3]. Our findings reveal distinct advantages of WPI-PG hydrogels as multifunctional scaffolds for tissue engineering, as they support stem cell adhesion, extracellular matrix formation, inhibition of cancer cell growth and adhesion, and, importantly, they possess antibacterial properties. The exploration of the use of WPI-PG hydrogels in bone tissue engineering would necessitate more experimental data on the *in vitro* and *in vivo* osteogenic potential of these materials.

It was observed that the proliferation and viability of MG63 cells were lower at the highest concentrations of PG (10%, 20%), which also showed their highest antimicrobial activity. It is possible that one must strike a balance between antimicrobial activity and cell proliferation and viability. Interestingly, the reduction in proliferation and viability was not as pronounced for DPSCs. It may be speculated that MG63 cells, being osteosarcoma-derived, are more susceptible to the anticancer effect of PG, which has been reported previously for phlorotannins, of which PG is the fundamental subunit [53]. This study underlines the suitability of WPI hydrogels as scaffolds for regeneration of bone tissue, as bone-forming cells were able to grow on WPI hydrogels, as in previous studies [6, 38]. Other authors have developed hydrogels for bone regeneration, both injectable and pre-formed, incorporating a mineral phase to combine the benefits of a hydrogel and a ceramic phase [54]. The WPI hydrogels in this study are pre-formed, and hence non-injectable, but they displayed values of Young's modulus of between 3 and 4 MPa (Fig. 3), which is considerably higher than values described for injectable hydrogels; for example, hydrogels of catechol/poly(ethylene glycol) (cPEG) and oligo[poly(ethylene glycol) fumarate] (OPF) used in previous work displayed Young's modulus values two orders of magnitude lower [55]. Stiffer surfaces are known to promote differentiation of cells toward the osteoblastic phenotype [56, 57]. Future work may focus on the incorporation of a mineral phase into WPI-PG hydrogels to exploit the positive biological effects of PG described in this study.

5. Conclusions

In this study, WPI hydrogels were enriched with PG at concentrations of 0, 2.5, 5, 10 and 20% (w/v). Incorporation of PG into WPI hydrogels increased the solubilization of PG. The addition of PG into the WPI hydrogels did not lead to any decrease of the mechanical properties as evidenced by the compressive strength, Young's modulus and % strain at break. The WPI-PG hydrogels demonstrated antimicrobial activities against a range of bacteria causing common HAI. WPI-PG hydrogels supported the growth and collagen production of DPSCs for the formation of the extracellular matrix. Overall, WPI modification with PG supported mesenchymal stem cell adhesion and growth, inhibited cancer cell growth and adhesion, demonstrating a bactericidal and bacteriostatic inhibition of a range of pathogenic bacteria, thus presenting a promising strategy in the development of multifunctional biomaterials for tissue engineering.

Disclosure

The authors declare no potential conflicts of interest.

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Phloroglucinol-enhanced whey protein isolate hydrogels with antimicrobial activity for ~~bone~~ tissue engineering

Varvara Platania^{1,2}, Timothy E.L. Douglas^{3,4,*}, Mikhajlo K. Zubko⁵, Danny Ward⁶, Krzysztof Pietryga⁷, Maria Chatzinikolaidou^{1,2,*}

¹ Dept. of Materials Science and Technology, University of Crete, 70013 Heraklion, Greece

² Foundation for Research and Technology Hellas (FORTH)-Institute of Electronic Structure and Laser (IESL), 70013 Heraklion, Greece

³ Engineering Department, Lancaster University, UK

⁴ Materials Science Institute (MSI), Lancaster University, UK

⁵ Centre for Bioscience, Manchester Metropolitan University (MMU), Manchester, UK

⁶ Department of Biological and Life Sciences (BLS), Lancaster University, UK

⁷ Department of Biomaterials and Composites, AGH University of Science and Technology, Krakow, Poland

* Corresponding authors

Maria Chatzinikolaidou, mchatzin@materials.uoc.gr

Department of Materials Science and Technology, University of Crete, 70013 Heraklion, Greece

Tel. +30 2810394276

Timothy Douglas, t.douglas@lancaster.ac.uk

Engineering Department, Lancaster University, UK

ABSTRACT

Aging populations in developed countries will increase the demand for implantable materials to support tissue regeneration. Whey Protein Isolate (WPI), derived from dairy industry by-products, can be processed into hydrogels with the following desirable properties for applications in tissue engineering: (i) ability to support adhesion and growth of cells; (ii) ease of sterilization by autoclaving and (iii) ease of incorporation of poorly water-soluble drugs with antimicrobial activity, such as phloroglucinol (PG), the fundamental phenolic subunit of marine polyphenols.

In this study, WPI hydrogels were enriched with PG at concentrations between 0 and 20% w/v. PG solubilization in WPI hydrogels is far higher than in water. Enrichment with PG did not adversely affect mechanical properties, and endowed antimicrobial activity against a range of bacteria which occur in healthcare-associated infections (HAI). WPI-PG hydrogels supported the growth of, and collagen production by human dental pulp stem cells and - to a lesser extent - of osteosarcoma-derived MG-63 cells. In summary, enrichment of WPI with PG may be a promising strategy to prevent microbial contamination while still promoting stem cell attachment and growth.

Keywords: whey protein, phloroglucinol, hydrogels, osteogenic differentiation, cytocompatibility, dental pulp stem cells, MG-63 cells, antimicrobial activity

1. Introduction

Treatment of critical size bone defects generally requires surgeries involving implantation of autologous bone grafts or bone graft substitutes. Tissue engineering (TE) aims to develop innovative scaffolds for the regeneration of tissues. The approach of delivering stem cells to the defect site on scaffolds with osteoconductive properties to enhance bone healing has attracted great attention. The formation of new bone tissue depends on the osteogenic development of appropriate stem cells [1]. Besides the great efforts to treat large critical-sized bone defects, few clinical applications are available due to the limitations of materials and low functionality of the platforms [2] (REFS). Hydrogels possess great advantages in bone repair, as they are composed of three-dimensional hydrophilic polymer chains, which have superior mechanical strength and can provide nutrient environments suitable for endogenous cell growth. They are able to mimic the natural ECM, thus presenting a prospective ability to encapsulate bioactive molecules or cells. Additionally, raw materials for preparation of hydrogels are extensive and readily available, and they can be tailored to obtain the desired geometry for implantation or injection, and the degradation rate and porosity can be easily controlled by altering the crosslinking method and degree.

Recent advances in tissue engineering have focused on novel multifunctional biomaterials [3] based on renewable resources of great natural abundance, low cost and safety for human health. Protein-based eco-friendly biomaterials such as dairy proteins are currently of interest as substitutes for commercial materials for biomedical applications [4]. Whey proteins represent 20% of total milk proteins and are generally generated as a by-product of cheese manufacture. They are unique from a nutritional point of view as they contain all the essential amino acids, and they contain β -lactoglobulin, α -lactalbumin, immunoglobulins and bovine serum albumin [5, 6]. In addition, antimicrobial activity in milk is mainly attributed to some minor whey proteins such as lactoferrin, lysozyme, immunoglobulins, and lactoperoxidase [7, 8].

Furthermore, WPI solutions can form hydrogels upon heating; an important practical advantage of WPI hydrogels is that they withstand sterilization by autoclaving, a commonly used and clinically accepted sterilization technique. Hydrogels display several advantages when compared to other materials used for TE, including solid polymer scaffolds and ceramic scaffolds: they are non-brittle and incorporation of biologically active substances is straightforward.

Polyphenols have been implicated as components of antimicrobial hydrogels [9, 10]. Polyphenols are derived from plants and are known to possess antibacterial effects and antioxidative properties. This is beneficial in bone TE, as oxidative stress has been indicated to impede both the differentiation of osteoblasts and the resorption of bone by osteoclasts *in vitro* [11, 12]. The ability to ameliorate oxidative stress by phenolic compounds such as gallic acid isolated from terrestrial plants, and phloroglucinol (PG) found in seaweeds has been documented in numerous reports [13, 14]. PG has also been found to inhibit metastatic ability of breast cancer cells *in vitro* and *in vivo* [15]. The antibacterial effect of phlorotannins, polyphenols derived from brown seaweed of which PG is the basic subunit, is well-known [16], while recent research suggests that PG in its different forms also possesses antimicrobial activity [17, 18]. Healthcare-associated infections (HAI) are a burden worldwide, especially due to the increasing prevalence of antibiotic-resistant strains. Hence, antimicrobial activity is an added value for biomaterials used in TE applications.

Bone marrow (BM)-derived mesenchymal stem cells (MSCs) are the major cell source for bone TE. However, the morbidity of the donor site and painful procedures for harvesting these cells have limited their application [19]. Recently, human dental pulp stem cells (DPSCs) were introduced as an alternative to multipotent stem cells with self-renewal ability [20]. These cells could be harvested from both primary and permanent teeth during routine teeth extraction procedures in orthodontic treatment [20]. For bone TE, DPSCs showed a higher proliferative ability and number of colony clusters compared to BM-MSCs

[21]. Although other sources of MSCs have been applied for the regeneration of hard tissues, the complications at the site of harvest have limited their applications in practice. The DPSCs are an easily accessible source of MSCs with similar characteristics to bone marrow-derived stem cells and therefore can be used for hard tissue regeneration [22]. Due to their availability and differentiation potential, the application of these cells for TE has attracted enormous attention in recent years, including bone TE studies in animal models [21-24].

MG-63 cells derived from human osteosarcoma have been widely used in bone biology research to assess the *in vitro* osteogenic potential of biomaterials for bone TE and the pharmacokinetics for bone disease, as an alternative to normal osteoblastic cells due to ease of their availability and reproducibility of experimental results [25, 26]. MG-63 cells together with Saos2 and MC3T3-E1 cell lines are used as reliable and adequate cell culture models that resemble the behavior of primary osteoblasts. However, differences in phenotypic characteristics, proliferative capacity, secretion of osteogenic proteins and gene expression levels in MG-63 and osteoblasts have been reported, including the expression of abundant amounts of type III collagen and fibronectin in MG-63 cells [27]. The latter are constituents of soft connective tissue and absent in mineralized bone. Despite these differences, the MG-63 cell line is a useful cell model for *in vitro* biological assessment, including the evaluation of cell attachment, proliferation and osteogenic differentiation.

In this paper, DPSCs and MG-63 cells were cultured in the presence of WPI-PG hydrogels, namely WPI hydrogels modified with different concentrations of PG, and were evaluated for their viability, proliferation, cell adhesion, morphology, potential to enhance extracellular collagen production and antimicrobial properties. As this potential is expected to be different for cell lines and primary cells, physiological and cancer cell types, a comparative *in vitro* biocompatibility study was performed. Viability and proliferation of both cell types on the WPI hydrogels were quantified by means of the PrestoBlue® cell viability assay. Cell adhesion and morphology of both cell types on WPI-PG hydrogels were visualized by means of scanning electron microscopy (SEM). Moreover, the collagen levels in culture supernatants were determined as a marker of extracellular matrix (ECM) [formation](#). Ten bacterial strains involved in [healthcare associated infections \(HAI\)](#), including Gram-positive and Gram-negative bacteria, [were selected](#) to perform sensitivity assays with WPI-PG hydrogels and evaluate their antimicrobial effects. All these strains present various types of primary and opportunistic HAI as well as food-borne and environmental infections. Seven of these strains belong to species included in the recently published list of drug-resistant bacteria that pose great health threats [28].

2. Materials and Methods

2.1 Hydrogel preparation

Stock solutions of 40% WPI (w/v) were prepared by dissolving 20 g of WPI powder in 50 ml of deionised H₂O. A sonicator was used to facilitate dissolution and minimize foaming. Subsequently, 15 ml of WPI stock solution was transferred to Falcon tubes, and the required amount of phloroglucinol (Sigma-Aldrich, Germany) was added. The WPI solutions enriched with 0 (control), 2.5, 5, 10 and 20% w/v PG concentrations are designated as WPI-PG-0%, WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10%, and WPI-PG-20%, respectively (Table 1). The WPI-PG solutions were vortexed until mixed homogeneously, and 15 ml were poured into 9 cm (in diameter) Petri dishes and autoclaved at 121°C for 15 minutes to simultaneously sterilise and induce gelation. Samples were then cooled to 60°C and cooled further at ambient temperature. The WPI-PG hydrogels were cut into disc-shaped samples by means of a biopsy punch with a diameter of 8 mm and a weight of approximately 50 mg.

Table 1. WPI hydrogels enhanced with different concentrations of PG. The WPI content of 40% (w/v) was the same for all WPI-PG hydrogels.

Material designation	PG content (%w/v)
WPI-PG-0% (control)	0
WPI-PG-2.5%	2.5
WPI-PG-5%	5
WPI-PG-10%	10
WPI-PG-20%	20

2.2 Surface topography studies

Sample cross-sections comprising a range of WPI-PG hydrogels were viewed by SEM. SEM analysis was performed using a 7800F JEOL SEM instrument. Prior to SEM analysis, samples were mounted on aluminum stubs (Agar Scientific Ltd, UK) using carbon adhesive pads (Agar Scientific Ltd, UK) and coated with a 10 nm layer of gold using a Q150RES sputter coater (Quorum Technologies Ltd). SEM was used to analyze differences in the surface topography of WPI-hydrogels modified with PG concentrations ranging from 0 to 20%.

2.3 Mechanical testing

The effects of PG incorporation into WPI hydrogels on the hydrogels' mechanical properties were evaluated. Cylindrical WPI samples of 2.5, 5, 10 and 20% PG, measuring 10 mm in height and 8 mm in diameter, were subject to compressive testing using a Zwick 1435 Universal Testing Machine. Test parameters were set at a speed of 10 mm per minute, and a test range of 0-95% strain.

2.4 DPSCs and MG-63 cell culture maintenance

DPSCs cultures were established after generous donation by Prof. A. Bakopoulou at the School of Dentistry at the Aristotle University of Thessaloniki, Greece. Based on a previously described protocol [22], DPSCs cultures were established using an enzymatic dissociation method following their isolation from wisdom teeth of healthy donors after informed consent according to the Declaration of Helsinki (ethical approval number:

322/15-4-2013 by the Institutional Ethics Committee). Briefly, following disinfection of third molars, a cut was made around the cementum-enamel junction to expose the pulp chamber. Then, the pulp was retrieved, thoroughly minced and digested for 45 min at 37°C with a mixture of 3 mg/ml of collagenase I and 4 mg/ml of dispase II (Invitrogen, Karlsruhe, Germany). DPSCs were cultured in minimum essential media α -MEM (Invitrogen) supplemented with 15% fetal bovine serum (FBS, Invitrogen), 100 mM L-ascorbic acid phosphate (Sigma-Aldrich, Steinheim, Germany), 100 units/ml of penicillin, 100 mg/ml of streptomycin and 1% amphotericin as previously described by the same team [22]. All experiments were conducted with DPSCs at passages 2 to 4.

The osteosarcoma cell line MG-63 was purchased from Sigma (USA) and cultivated in DMEM containing 100 units/ml of penicillin, 100 mg/ml of streptomycin and 1% amphotericin (all from Gibco), supplemented with 10% fetal bovine serum (FBS) according to bibliography [29-31]. Cells were maintained in a humidified atmosphere under 5% CO₂ at 37°C, and the media were changed twice a week.

2.5 Cell viability and proliferation assay

The viability and proliferation of DPSCs and MG-63 on WPI-PG hydrogels was quantitatively assessed using the resazurin-based metabolic assay PrestoBlue® (Invitrogen, USA) as previously described [32]. Briefly, the disc-shaped WPI-PG scaffolds, generated by using an 8 mm biopsy cutter, were placed into 48-well plates and each sample was seeded with 2×10^4 cells. At each experimental time point of 2, 4 and 7 days of culture, the PrestoBlue® reagent was added directly to the wells at a 1:10 ratio in culture medium and incubated at 37°C for 60 min, before measuring the absorbance at 570 and 600 nm in a spectrophotometer (Synergy HTX Multi-Mode Microplate Reader, BioTek, Winooski, VT, USA). Three independent experiments were performed in triplicate.

2.6 Adhesion and morphology of the DPSCs and MG-63 cells on the WPI-PG hydrogels

The adhesion and morphology of DPSCs and MG-63 osteoblast-like cells were observed using SEM (JEOL JSM-6390 LV) after 2, 4 and 7 days in culture. Seeded scaffolds with 2×10^4 cells per sample were placed in a CO₂ incubator at 37°C for 2, 4 and 7 days and then were removed from the incubator and rinsed three times with PBS, fixed with 4% v/v para-formaldehyde for 20 min and dehydrated in increasing concentrations (30–100% v/v) of ethanol. The scaffolds were then dried in a critical point drier (Baltec CPD 030), sputter-coated with a 20 nm thick layer of gold (Baltec SCD 050) and observed under SEM at an accelerating voltage of 15 kV.

2.7 Determination of the produced extracellular collagen

A modified Sirius red assay [33] was used to stain total collagen produced in the extracellular matrix. The anionic dye Sirius Red binds to the [Gly–X–Y] tripeptide of mammalian cells. Collagen is a basic protein, and it is thus likely that the sulphonic groups of the dye may interact at low pH with the amino groups of lysine and hydroxylysine, and with the guanidine groups of arginine [34]. On days 2, 4 and 7 of the experiment, the culture supernatants were collected, and 25 μ l were diluted to a final volume of 100 μ l with ultrapure water. Then 1 ml of the dye solution consisting of 0.1% w/v Sirius red F3B (Sigma-Aldrich, St. Louis, MO, USA) in 0.5 M acetic acid was added, and the samples were incubated at room temperature for 30 min. The samples were centrifuged at 15,000 g for 20 min to pellet the collagen–dye complex. The pellet was washed with 0.5 ml of 0.5 M acetic acid to remove the unbound dye. Dissolution of the collagen–dye complex with 1

ml of 0.5 M NaOH followed, and 200 μ l of the solution were transferred into 96-well plates to measure the absorbance at 530 nm (Synergy HTX Multi-Mode Microplate Reader, BioTek, Winooski, VT, USA). Collagen concentration was determined by means of a calibration curve. Samples were analyzed in quadruplicate.

2.8 Microbiological assays

Bacterial strains used in the study included *Bacillus cereus* (MCIMB 9373), *Enterococcus faecalis* (NCTC 12697), *Escherichia coli* (NCTC 9001), *Klebsiella oxytoca* (ATCC 15764), *K. pneumoniae* (40602), *Pseudomonas aeruginosa* (NCTC 6749), *Staphylococcus aureus* (NCTC 6571), *S. aureus* (MRSA) [originated from NCTC 13552], *S. epidermidis* (NCTC 11047), *Streptococcus pyogenes* (NCIMB 8198).

For susceptibility testing, the bacterial strains were grown overnight on nutrient agar (Oxoid CM0003) at 37°C. Inocula were prepared according to the standard protocol (Andrews and BSAC Working Party On Susceptibility Testing ft 2001). Disc-shaped WPI-PG hydrogels were prepared and placed onto a bacterial inoculum. For sensitivity tests, the samples were placed on the surface of plates containing 25 ml of Iso-sensitest agar (Oxoid CM0471) or (in some experiments) nutrient agar. Before placing the samples, the agar plates were swabbed with microbial suspensions. The plates were incubated overnight at 37°C. Zones of inhibition (ZOI) were recorded after 18-30 h when bacterial lawns became clearly visible on the agar.

To test modes of inhibition of bacterial growth, the samples of hydrogels were removed from ZOI using sterile forceps, and central areas of ZOI without any signs of bacterial growth were touched firmly a few times with sterile wet swabs, streaked out (in single lines) onto sectors on nutrient agar plates (Supplementary Fig. 2), and incubated overnight at 37°C. The absence of bacterial growth on the streaked areas was interpreted as bactericidal inhibition; the restoration of growth indicated bacteriostatic effects.

2.9 Statistical analysis

The experimental data were analyzed using two-way ANOVA followed by Tukey's multiple comparisons test among groups. Data were expressed as means \pm standard deviation (SD). For these analyses the GraphPad Prism software version 8.0 (La Jolla, USA) was used. The symbols * designate $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns denotes statistically non-significant differences.

3. Results

3.1 WPI-PG hydrogels and surface characterization by means of SEM

Sterile, disc-shaped WPI-PG hydrogels were prepared as described in section 2.1, and based on their PG concentration they were denoted as WPI-PG-0% (control), WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10%, and WPI-PG-20%. The WPI-PG hydrogels changed colour from yellowish for WPI-PG-0% to dark brown for the WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10%, and WPI-PG-20% (Fig. 1).

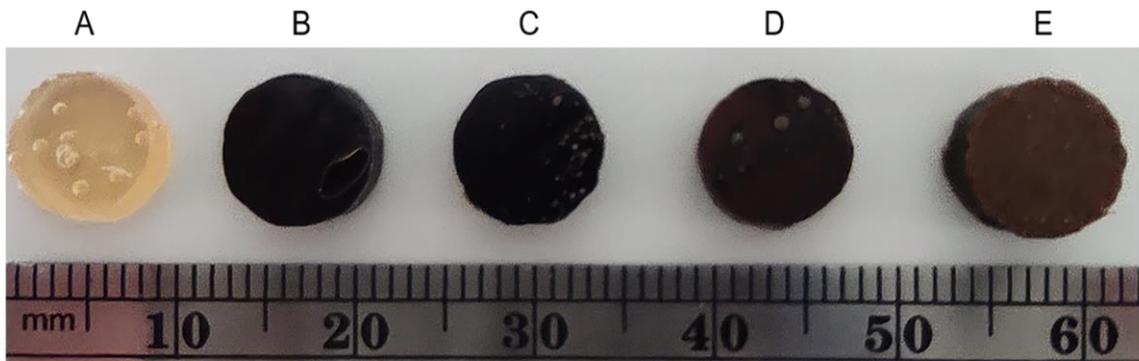


Fig. 1: Macroscopic images of disc-shaped samples prepared from hydrogels solidified and autoclaved in petri dishes. A: WPI-PG-0%, B: WPI-PG-2.5%, C: WPI-PG-5%, D: WPI-PG-10%, E: WPI-PG-20%.

The microstructures of WPI-PG hydrogels with concentrations ranging from 0 to 20% PG were analyzed via SEM (Fig. 2). The WPI control (0% PG) sample presented a consistently smooth surface topography. It is conceivable that the increase in roughness is caused by the presence of PG in the hydrogel, which may form deposits when hydrogels are dried during preparation for SEM studies.

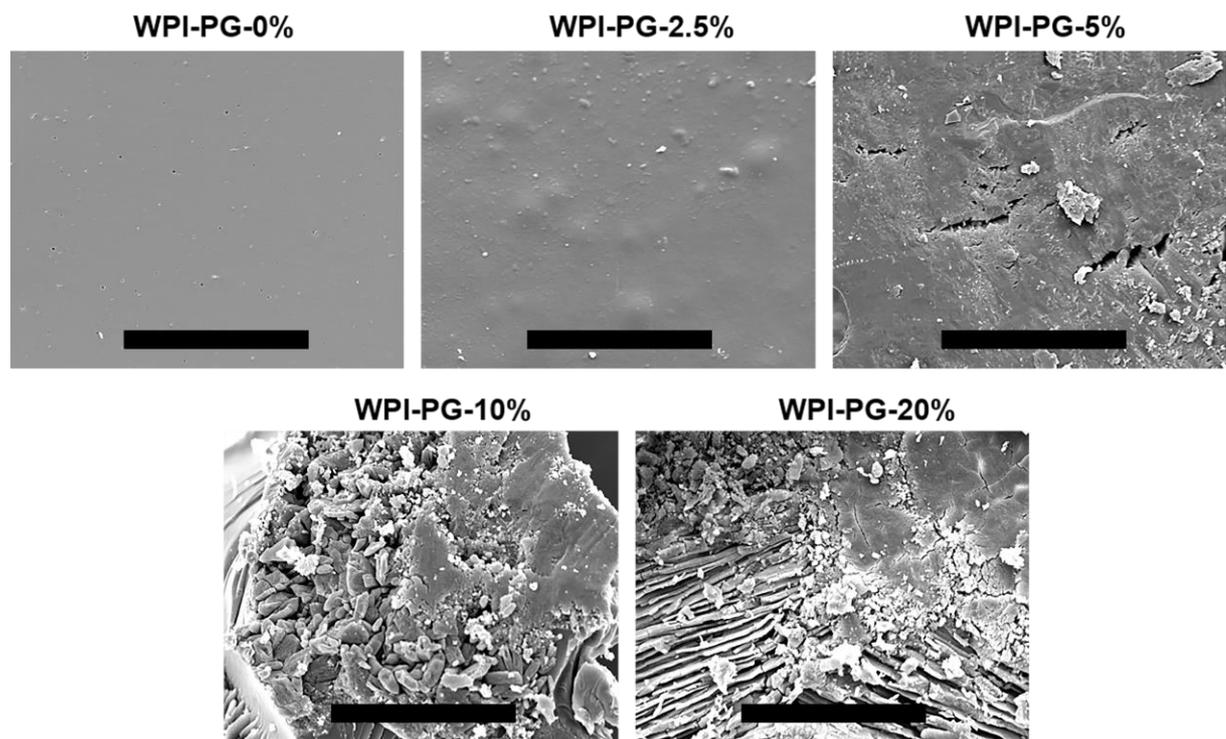


Fig. 2. SEM images displaying alterations in surface topography of WPI hydrogels containing PG at concentrations of 0%, 2.5%, 5%, 10% and 20%. Scale bars on SEM images indicate 1000 μm .

3.2 Mechanical testing

The WPI-PG-0%, WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10% and WPI-PG-20% hydrogels were characterized for mechanical properties by measuring their compressive strength,

Young's modulus and % strain at break (Fig. 3). It is important to note that, due to high friction caused by the nature of the samples, the WPI hydrogels supplemented with 20% PG became stuck in the jaws of the Zwick testing machine, meaning values for strength, modulus and strain at break may have been overestimated.

3.2.1 *Compressive strength*

The compressive strength values for WPI hydrogels containing 0, 2.5, 5, 10 and 20% PG are shown in Fig. 3A. No statistically significant difference in compressive strength was observed between the WPI control (0% PG) and the 2.5% PG sample group (mean values of 4.26 and 4.34 MPa, respectively). However, increasing the PG concentration to 5% resulted in the compressive strength decreasing by 1.35 MPa to a mean of 2.91 MPa. This reduction was deemed to be statistically insignificant compared to both the WPI control and the 2.5% PG sample group ($P < 0.05$). Increasing the PG concentration to 10% led to a mean compressive strength of 5.06 MPa. However, values were inconsistent within this group and no statistical significance was proven. Values for the 20% PG sample group were significantly higher by a factor of approximately three compared to the other groups; however, these values may have been overestimated.

3.2.2 *Young's modulus*

Except for the 20% PG sample group for which values may have been overestimated, no statistically significant difference in Young's modulus was observed between PG supplemented sample groups compared to the WPI control (Fig. 3B). The addition of 2.5% PG resulted in a negligible increase in Young's modulus, while 5% PG caused a negligible decrease. A statistically significant increase of 0.43 MPa was observed when PG concentration was increased from 5% to 10%. However, all samples demonstrated a Young's modulus between 3 and 4 MPa.

3.2.3 *Strain at break*

The % strain at break of the WPI samples with different PG concentrations is presented in Fig. 3C. The mean deformation observed for the WPI control (0% PG) sample group was 7.09 mm (70.92%). The presence of 2.5% PG in WPI hydrogels resulted in a slight increase in the strain at break (72.31%) while supplementation of 10% decreased this value to 70.14%. However, except for the potential overestimate in the 20% PG samples, the only statistically significant difference ($p < 0.05$) observed for strain at break coincided with the addition of 5% PG (61.78%).

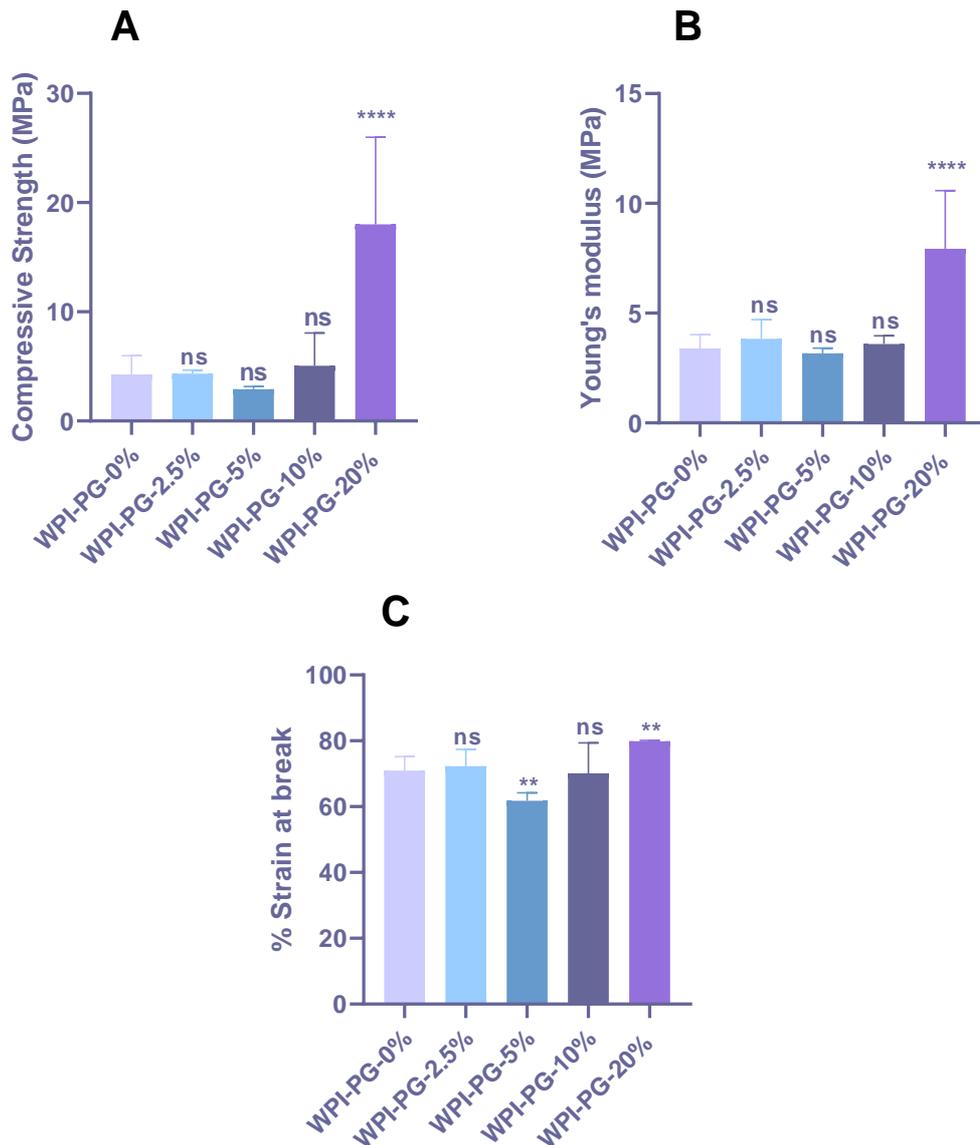


Fig. 3. Mechanical testing of WPI hydrogels containing 0, 2.5, 5, 10 and 20% PG. A: Compressive strength; B: Young's modulus; C: Strain at break. Each bar represents the mean \pm SD of nine replicates (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$; ns denotes statistically non-significant difference compared to the WPI-PG-0 control).

3.3 Cell viability and proliferation

Cellular viability and proliferation of DPSCs and MG-63 on WPI-PG-0%, WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10% and WPI-PG-20% hydrogels were quantitatively assessed by measuring their metabolic activity after 2, 4 and 7 days in culture (Fig. 4). For the whole incubation period, cell numbers for both cell types investigated were found to be higher on the tissue culture treated polystyrene (TCPS) control than on the WPI-PG hydrogels (Fig. 4_A, C).

DPSCs displayed increased metabolic activity, expressed as absorbance value, from day 4 to day 7 in all samples, with the WPI-PG-10% and WPI-PG-5% hydrogels demonstrating non-significant difference to TCPS control during day 4 (Fig. 4_A). As compared with DPSCs, MG-63 osteosarcoma cells presented a decrease of metabolic activity from day 4

to day 7 in all samples, indicating a downregulated proliferation potential of osteosarcoma cells on WPI-PG hydrogels.

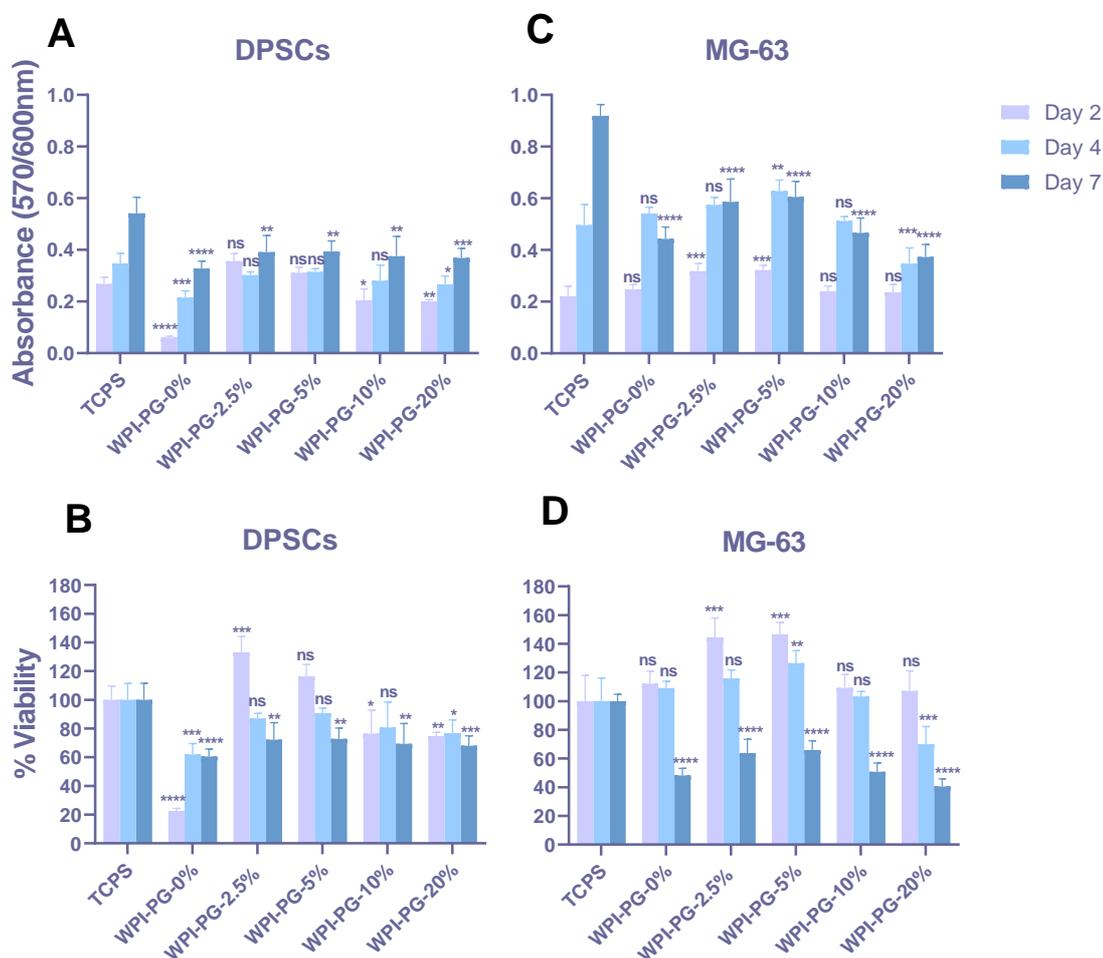


Fig. 4. Cell viability and proliferation of DPSCs (A, B) and MG-63 (C, D) cells cultured in the presence of WPI hydrogels with different PG concentrations, expressed as absorbance (A, C) and % viability of the TCPS control set at 100% for each experimental time point (B, D). Each bar represents the mean \pm SD of quadruplicates (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns denotes a statistically non-significant difference compared to the TCPS control at the corresponding time point).

3.4 Effects of WPI-PG hydrogels on cell adhesion, morphology and growth

Representative SEM images show DPSCs on WPI-PG-0%, WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10% and WPI-PG-20% hydrogels, 4 and 7 days post-seeding (Fig. 5). Images at days 4 and 7 showed cell spreading for increased PG concentrations in WPI-PG-10% and WPI-PG-20% hydrogels with DPSCs, however without displaying their characteristic spindle shape morphology until day 7. On day 7 in culture, DPSCs displayed cell-cell interactions that promote tissue formation. Cell infiltration within the pores of the WPI-PG-

10% and WPI-PG-20% hydrogels was observed ~~as indicated with white arrows~~ in Fig. 5. In contrast, MG-63 cells appear round on the surface of the hydrogels with an obvious decrease in cell density on WPI-PG-2.5% and WPI-PG-5% hydrogels (Fig. 6). On the latter two compositions the observed cell density of DPSCs was lower as well.

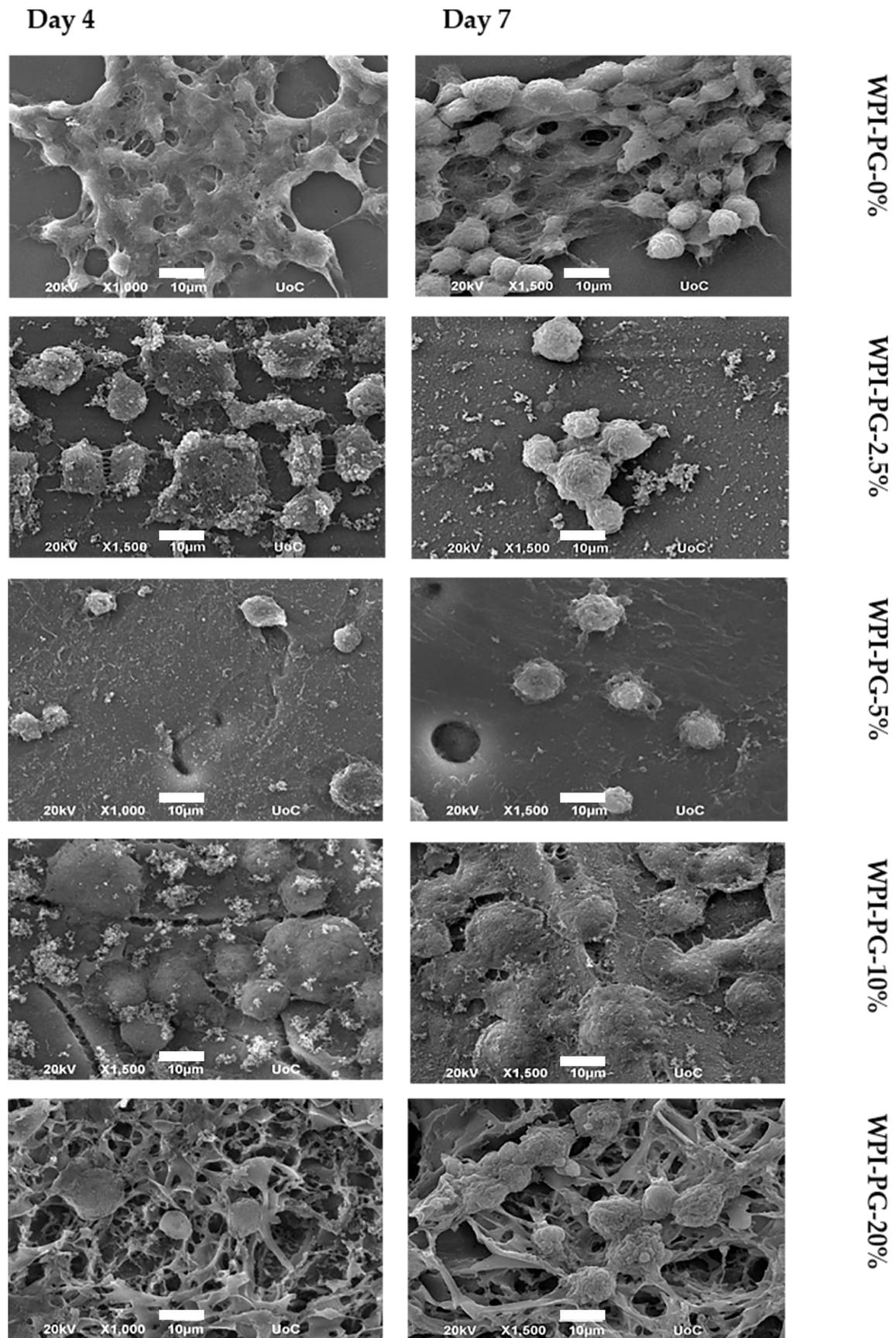


Fig. 5. Evaluation of cell attachment and morphology of DPSCs cultured on WPI hydrogels modified with different PG concentrations after 4 and 7 days in culture. Scale bars represent 10 µm.

The morphology of MG-63 cells on WPI-PG-0%, WPI-PG-2.5%, WPI-PG-5%, WPI-PG-10% and WPI-PG-20% hydrogels on day 4 and 7 was visualized via SEM and presented in Fig. 6. WPI-PG-10% and WPI-PG-20% hydrogels promoted cell spreading.

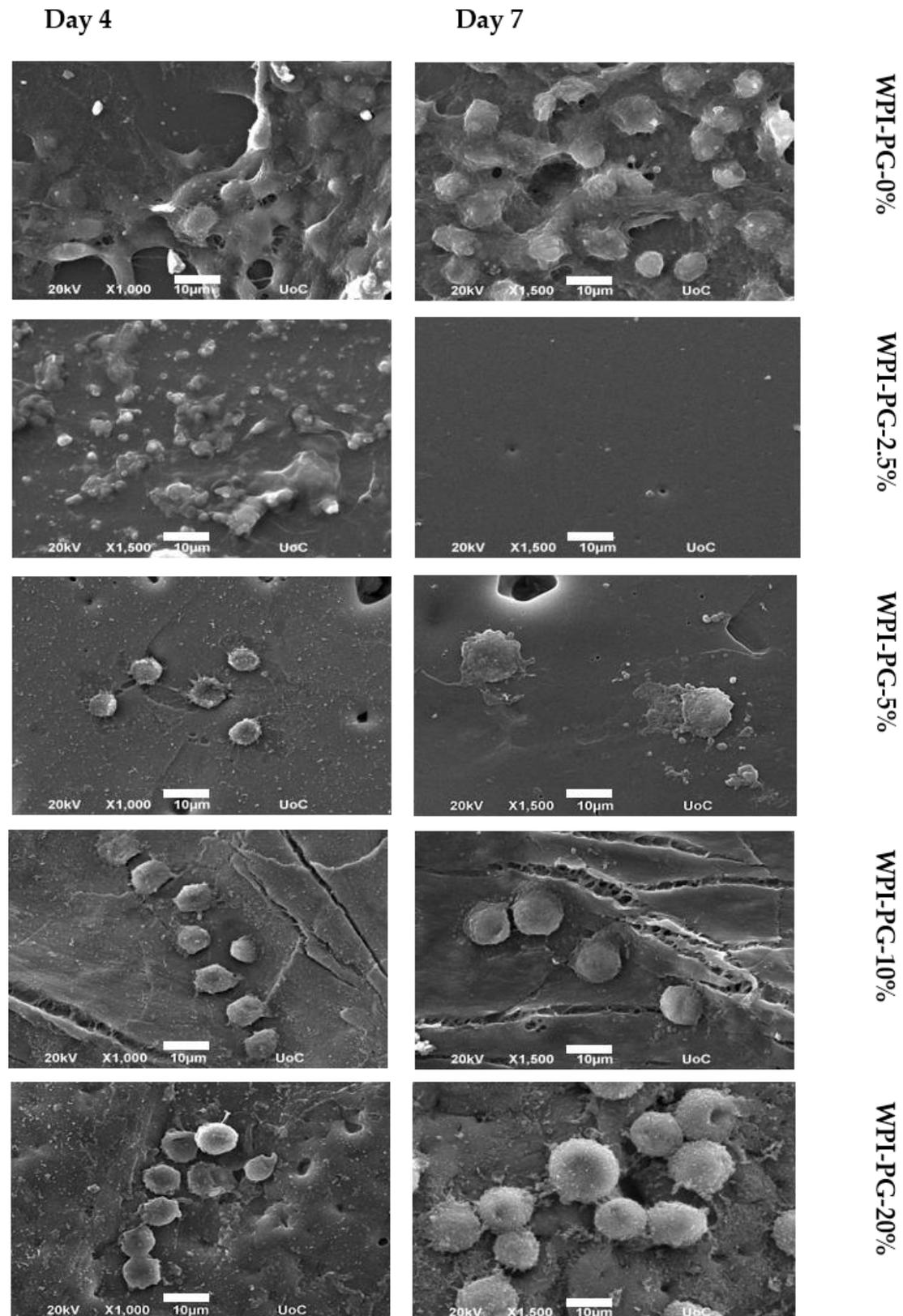


Fig. 6. Evaluation of cell attachment and morphology of MG-63 osteosarcoma cells cultured on WPI hydrogels modified with different PG concentrations, after 4 and 7 days in culture. Scale bars represent 10 μm .

3.5 Extracellular collagen production

In order to investigate the potential of ECM formation by DPSCs and MG-63 cultured on the WPI-PG hydrogels, the secretion of collagen was determined, as collagen constitutes the main structural element of the ECM. We quantified the total collagen secreted in culture supernatants at different time intervals, as shown in Fig. 7, in the presence of exogenous ascorbic acid which is a co-factor in collagen synthesis [35]. We found that DPSCs produced similar levels of collagen on days 2 and 4 in the presence of all WPI-PG hydrogels. On day 7, significantly lower values were observed for WPI-PG-2.5%, WPI-PG-5%, and WPI-PG-10% compared to the WPI-PG-0% control, while for WPI-PG-20% they were the highest among the hydrogels and similar to the control. MG-63 osteosarcoma cells exhibit significantly lower levels of collagen production compared to the DPSCs cultures. Collagen production levels in MG-63 cultures were similar to the WPI-PG-0% control on days 2 and 4, except of the WPI-PG-20% hydrogels that indicated significantly higher values than the control on day 2.

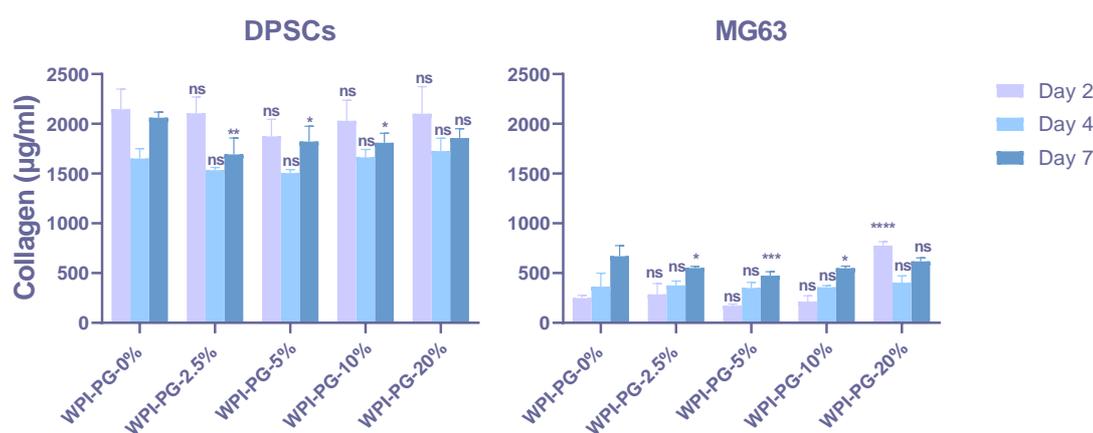


Fig. 7. Levels of total collagen in the supernatants of the DPSCs (left) and the MG-63 cells (right) cultured on the different PG concentrations in WPI-hydrogels for 2, 4 and 7 days. Error bars represent the average of quadruplicates (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns denotes statistically non-significant difference compared to the WPI-PG-0% control at the corresponding time point).

3.6 Antibacterial properties of WPI-PG hydrogels

First assays involving testing of antimicrobial properties of disc-shaped WPI-PG hydrogels were carried out against a MRSA strain. The examples of these experiments are shown in Supplementary Fig. 1. It is evident from Supplementary Fig. 1a that 5 mg of the WPI-PG-2.5%, WPI-PG-5% and WPI-PG-10% hydrogels did not inhibit the MRSA growth, but there was slight growth inhibition with a narrow ZOI in WPI-PG-20%. The assay involving 20 mg

of WPI-PG-20% showed a clear ZOI. To enhance the assay sensitivity, the amount of the hydrogels was increased to 30 mg in the next experiment (Supplementary Fig. 1b), in which five bacterial species (*Pseudomonas aeruginosa*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Escherichia coli* and *Streptococcus pyogenes*) manifested clear ZOI, and one species (*Enterococcus faecalis*) was marginally inhibited in its growth. These results suggest an obvious antibacterial potential of the hydrogel at least against seven bacterial species. Moreover, disc-shaped hydrogel samples were used for the sensitivity testing, and the results are shown in Fig. 8. All four bacterial species used in this experiment were clearly inhibited in their growth by WPI-PG hydrogels at all used concentrations of PG on two different media (Iso-sensitest and nutrient agar). The level of inhibition (ZOI size) was dependent on PG concentrations. Overall, the inhibitory effects were stronger and clearer on Iso-sensitest agar than on nutrient agar, although the results for *P. aeruginosa* were comparable on both media. The blank WPI hydrogels without PG did not manifest any inhibitory effects.

After recording the results on sensitivity testing (next day following swabbing), we reused the plates with ZOI for determining the mode of growth inhibition in a simple assay (see Methods). Supplementary Fig. 2 shows an example of results from such assay performed for WPI-PG-10% and WPI-PG-20% hydrogels within the experiment depicted in Fig. 8. The mode of inhibition depended on PG concentrations and media used. Bactericidal growth inhibition was observed for WPI-PG-20% hydrogels in *P. aeruginosa* on both media, and in *S. epidermidis* on Iso-sensitest, as well as for WPI-PG-10% hydrogels in *S. epidermidis* on Iso-sensitest. Predominantly bactericidal inhibition with a bacteriostatic effect for a low proportion of cells was observed for WPI-PG-20% hydrogels in MRSA on both media, and in *K. oxytoca* on nutrient agar. WPI-PG-10% hydrogels showed bacteriostatic inhibition on both media for MRSA, *K. oxytoca* and *P. aeruginosa*. Bacteriostatic inhibition was also observed for WPI-PG-20% hydrogels in *K. oxytoca* on Iso-sensitest, and in *S. epidermidis* on nutrient agar.

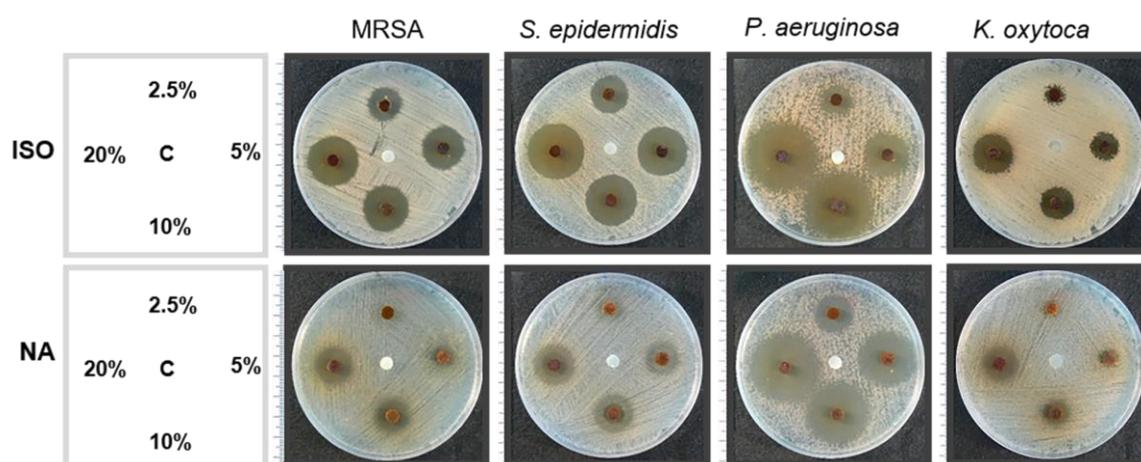


Fig. 8. Inhibition of bacterial growth by WPI-PG hydrogels on Iso-sensitest agar (ISO) and nutrient agar (NA). The squares on the left show the arrangement of hydrogel discs with different concentrations of PG and without PG (C). The ruler (with 1 mm scale) is attached to the left side of each image. The diameter of each plate is 9 cm.

4. Discussion

Recent advances in TE focus on finding more biocompatible, biodegradable and non-toxic biomaterials with the ability to mimic the natural physiological environment and favor enhanced cell-material interactions. Protein-based biomaterials have the characteristics of improved bioresorbability, biocompatibility, tissue healing, and ability to promote regeneration [4, 36-38]. In this work we report on WPI and PG for the development of multifunctional biomaterials for biomedical applications. PG was identified as a component of hydrogels due to its known antimicrobial and antioxidative properties. The investigation of biomaterials suitability for bone TE includes their capacity to promote cell proliferation, adhesion and ECM formation. We demonstrated that DPSCs and MG-63 showed different cellular responses when seeded on different WPI-PG hydrogels, which is in agreement with literature reports [25].

The microstructure of WPI hydrogels has been examined by means of SEM. As expected, the control WPI hydrogels (0% PG) displayed a homogeneously smooth surface topography in the dry state [6]. With increasing PG concentration, hydrogels were observably rougher in the dry state. This may be an advantage for their potential applications in bone TE, as both stiffer [39] and rougher [40] surfaces are known to promote the differentiation of cells towards the osteoblastic phenotype.

Minimal increases in both Young's modulus and percentage strain at break were found in hydrogels supplemented with PG at lower concentrations of 2.5%. Since stiffer surfaces are known to promote the differentiation of cells towards the osteoblastic phenotype, PG imparted stiffness would improve the compatibility of these hydrogels with bone tissue. However, this effect was not observed in a dose-dependent manner. The values for Young's modulus, compressive strength and percentage strain at break remained relatively consistent throughout sample groups, even when the concentration of PG was increased to 10%. This suggests that the incorporation of PG into the WPI hydrogels has no significant impact on the overall structural function of WPI hydrogels up to a PG concentration of 10%. Values for hydrogels containing 20% PG were higher; however, it is important to note that, due to high friction, the WPI hydrogels supplemented with 20% PG remained stuck in the jaws of the testing machine, and therefore it cannot be concluded that this PG concentration improves the aforementioned mechanical properties. As such, PG supplementation at higher concentration may alter the mechanical properties of WPI composites, resulting in 'stickier' hydrogels. In future work, one might consider strategies to reduce adhesion between samples and the testing machine jaws with minimal influence on mechanical properties, for example the insertion of a thin, non-adhesive piece of material between the jaws and the sample.

Discussion of the mechanisms for the effect (or lack of effect) of PG on WPI hydrogels' mechanical properties must remain speculative in view of the limited data available. It is known that phlorotannins interact with proteins, resulting in a dark brown colour [41], which was ascribed to non-covalent protein-phlorotannin interactions. One may expect similar interactions between proteins and PG, the fundamental subunit of phlorotannins, which would explain the brown color observed in this study (Fig. 1). Possibly, the number of crosslinks caused by interactions between WPI proteins and PG must exceed a certain number in order to markedly influence mechanical properties. The increased "stickiness" of hydrogels containing 20% PG might be due to interactions between PG bound to the WPI hydrogel network and surfaces; hydrogels containing polyphenol groups have exhibited increased adhesiveness [42]. Again, one can speculate that a certain PG concentration should be exceeded before such "stickiness" becomes appreciable. Alternatively, it is possible that at high PG concentrations, some PG may have "precipitated" as particles within the hydrogels after cooling, which might conceivably cause mechanical reinforcement. However, as already mentioned, such discussion must remain speculative.

Since materials for biomedical applications are potential sites for exogenous and endogenous infections, it is beneficial if they possess antimicrobial properties [43]. To evaluate them, we used a simple and convenient approach of making disc-shaped hydrogel samples allowing easy standardization of weight, volume, shape and application

of hydrogel materials to microbial inocula. These advantages of disc-shaped samples make them comparable in efficiency to paper discs used routinely in sensitivity assays involving liquids [44, 45]. Using these samples allows quantification of sensitivity data (Supplementary Fig. 3).

Overall, all ten Gram-positive and Gram-negative bacteria used in our study were inhibited (in bactericidal or bacteriostatic mode) by WPI-PG hydrogels, suggesting presumably non-specific inhibitory mechanisms. Interestingly, *Candida albicans* was not inhibited by WPI-PG hydrogels (as shown in Supplementary Fig. 4), suggesting that eukaryotic cells might be generally tolerant to PG, which is consistent with our results for human cells cultivated *in vitro*. Modes of inhibition were determined by using a simple “streak out assay” to check re-growth of inhibited bacterial cells taken directly from ZOI. This assay is convenient and rapid. Although WPI itself possesses antimicrobial properties [46], we never observed inhibition of bacterial growth by WPI hydrogels without PG, probably due to the denaturation of proteins during hydrogels formation.

Ideally, an antibacterial biomaterial would exhibit both a burst release to prevent microbial colonization and help the recipient's cells win the “race to the surface”, and also a prolonged release to prevent later microbial colonization. For this, more detailed studies of PG release are necessary in future work. In addition, testing of antimicrobial properties of samples after incubation in biological fluids for a more extended period of time would be prudent to verify the mid- to long-term antimicrobial activity.

Regarding the cell viability assessment of the DPSCs and MG-63 cells on the WPI-PG hydrogels, we quantitatively evaluated the cytotoxic effect of substrates according to ISO 10993-5, in which it is stated that reduction of cell viability by more than 30% is considered a cytotoxic effect. In accordance, no cytotoxic effect was observed on days 2 and 4 for both cell types that displayed viability rates from 87-145% compared to the TCPS control, with higher values on WPI-PG-2.5% and WPI-PG-5% hydrogels on day 2. On day 7, the cell viability for DPSCs ranged from 60% to 73% compared to the control, while MG-63 cells presented viability values from 40% to 65%. Increased PG concentrations led to an inhibition of the MG-63 proliferation, consistent with the previously reported anti-cancer activity of PG [13, 15].

Collagen is the most abundant fibrous protein within the interstitial ECM that constitutes up to 30% of the total protein mass in mammals, providing tensile strength, regulating cell adhesion, supporting chemotaxis and migration, and directing tissue development [47]. Deposition of type I collagen-rich ECM is a prerequisite for the expression of specific osteoblast products, such as alkaline phosphatase, during the normal developmental sequence of the osteoblast [48]. Collagen levels secreted by DPSCs cultured on WPI-PG hydrogels are increased two to three-fold compared to the MG-63 secretion levels. These results suggest that WPI-PG hydrogels may have supported the ECM formation when seeded with DPSCs.

SEM micrographs of the 2D WPI-PG hydrogel surface underline the relationship between the increasing concentration of PG in the hydrogel and the supported adhesion and proliferation of DPSCs and MG-63 cells. In addition to cell adhesion, the morphology of the cells was evaluated. MG-63 osteosarcoma cells exhibited a round shape morphology in contrast to DPSCs, which were found to form cell-cell and cell-material interactions after 4 and 7 days in culture. Although MG-63 cells are widely used for biochemical studies of osteoblast-specific functions *in vitro*, their abundant type III collagen synthesis has been known to be unusual compared to other osteoblast lineages [49]. MG-63 osteosarcoma cells have been characterized as an atypical bone-forming model since the gene expression profile and the capability to synthesize a correct extracellular bone matrix are impaired [50]. It is also possible that the tumorigenic nature of MG-63 prevents them from properly adhering onto the WPI-PG hydrogels. In addition, the DPSCs culture contained ascorbic acid, a required cofactor for enzymes to hydroxylate proline and lysine in pro-collagen, enabling the collagen chains to form a proper helical structure [51]. The role of ascorbic acid in osteogenic differentiation is mainly attributed to the secretion of type I collagen into the ECM. The significantly higher total collagen production found in DPSCs

cultures on WPI-PG hydrogels compared to the MG-63 cells is in line with the cell-cell interactions observed for DPSCs by means of SEM indicating good adhesion and a high affinity to the substrate, similar to a previous study displaying strong attachment of DPSCs onto chitosan/gelatin scaffolds that enhanced bone tissue formation *in vitro* and *in vivo* [22]. The presence of cell-cell interactions suggests active cell motility, which is essential for tissue formation within a scaffold, and this is in agreement with a recent report on dense ECM formation by DPSCs cultured onto biopolymer-based hydrogels that promoted the osteogenic response *in vitro* [52]. Another study towards bone TE demonstrated the capacity of DPSCs to strongly attach on antibacterial thymol-functionalized hybrid scaffolds, increasing proliferation and calcium mineralization [3]. Our findings reveal distinct advantages of WPI-PG hydrogels as multifunctional scaffolds for tissue engineering, as they support stem cell adhesion, extracellular matrix formation, inhibition of cancer cell growth and adhesion, and, importantly, they possess antibacterial properties. The exploration of the use of WPI-PG hydrogels in bone tissue engineering would necessitate more experimental data on the *in vitro* and *in vivo* osteogenic potential of these materials.

It was observed that the proliferation and viability of MG63 cells were lower at the highest concentrations of PG (10%, 20%), which also showed their highest antimicrobial activity. It is possible that one must strike a balance between antimicrobial activity and cell proliferation and viability. Interestingly, the reduction in proliferation and viability was not as pronounced for DPSCs. It may be speculated that MG63 cells, being osteosarcoma-derived, are more susceptible to the anticancer effect of PG, which has been reported previously for phlorotannins, of which PG is the fundamental subunit [53]. This study underlines the suitability WPI hydrogels as scaffolds for regeneration of bone tissue, as bone-forming cells were able to grow on WPI hydrogels, as in previous studies [6, 38]. Other authors have developed hydrogels for bone regeneration, both injectable and pre-formed, incorporating a mineral phase to combine the benefits of a hydrogel and a ceramic phase [54]. The WPI hydrogels in this study are pre-formed, and hence non-injectable, but they displayed values of Young's modulus of between 3 and 4 MPa (Fig. 3), which is considerably higher than values described for injectable hydrogels; for example, hydrogels of catechol/poly(ethylene glycol) (cPEG) and oligo[poly(ethylene glycol) fumarate] (OPF) used in previous work displayed Young's modulus values two orders of magnitude lower [55]. Stiffer surfaces are known to promote differentiation of cells toward the osteoblastic phenotype [56, 57]. Future work may focus on the incorporation of a mineral phase into WPI-PG hydrogels to exploit the positive biological effects of PG described in this study.

5. Conclusions

In this study, WPI hydrogels were enriched with PG at concentrations of 0, 2.5, 5, 10 and 20% (w/v). Incorporation of PG into WPI hydrogels increased the solubilization of PG. The addition of PG into the WPI hydrogels did not lead to any decrease of the mechanical properties as evidenced by the compressive strength, Young's modulus and % strain at break. The WPI-PG hydrogels demonstrated antimicrobial activities against a range of bacteria causing common HAI. WPI-PG hydrogels supported the growth and collagen production of DPSCs for the formation of the extracellular matrix. Overall, WPI modification with PG supported mesenchymal stem cell adhesion and growth, inhibited cancer cell growth and adhesion, demonstrating a bactericidal and bacteriostatic inhibition of a range of pathogenic bacteria, thus presenting a promising strategy in the development of multifunctional biomaterials for tissue engineering.

Disclosure

The authors declare no potential conflicts of interest.

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