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Anti-Bacterial and Anti-Inflammatory Effects of a Ceramic Bone Filler Containing Polyphenols from Grape Pomace

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Abstract: Bone loss is a major burden for society and impacts people's health all over the world. In a changing world looking toward a more conscious use of raw materials, efforts are being made to increasingly consider new promising biomaterials that account for, on one side, the ability to provide specific functional biological activities and, on the other, the feature of being well tolerated. In this regard, the use of phenolic compounds in the field of bone-related bioengineering shows a rising interest in the development of medical solutions aimed at taking advantage of the multiple beneficial properties of these plant molecules. In this work, the anti-bacterial and anti-inflammatory power of a biphasic calcium phosphate synthetic bone filler coated with a mixture of phenolic compounds was investigated by evaluating the minimal inhibitory concentration (MIC) value against *Streptococcus mutans* and *Porphyromonas gingivalis* and the expression of genes involved in inflammation and autophagy by real-time reverse transcription polymerase chain reaction (RT-qPCR) on J774a.1 murine macrophage cells. Results show a MIC of 0.8 µg/mL, a neat anti-inflammatory effect, and induction of autophagy key genes compared to a ceramic bone filler. In conclusion, functionalization with a polyphenol-rich extract confers to a ceramic bone filler anti-bacterial and anti-inflammatory properties.

Keywords: anti-bacterial; anti-inflammatory; ceramic bone filler; calcium phosphates; polyphenols; periodontitis; periimplantitis; antioxidant; *S. mutans*; *P. gingivalis*



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1. Introduction

When talking about bone loss, one needs to consider different related diseases and disorders that can be grouped under the term bone diseases. The loss of bone mass during life is a normal phenomenon. In fact, the high prevalence of some of these skeletal diseases, especially osteoporosis and osteopenia, is due primarily to the aging of the population [1]. However, when the mechanisms of homeostasis that maintain the equilibrium between bone formation and bone resorption undergo instability and disruption by several conditions, such as genetic predispositions, the presence of certain diseases, the use of specific drugs, and an unhealthy lifestyle, pathologic bone loss appears [2], representing a chronic burden on both individuals and society [3].

In the oral context, this bone loss mainly occurs in association with periodontal diseases, which are thus considered among the most common medical conditions in the world [4]. In fact, the recently reported prevalence data show a global increase over the years that negatively impacts people's general health and quality of life [5]. Periodontal diseases are defined and characterized by a different degree of the host's inflammatory reactions induced by changes in the oral microbiome, that is, the loss of oral microbial diversity and beneficial microbes in favor of pathogenic bacteria, which can lead to a breakdown of the symbiotic relationship that develops with the host in eubiotic conditions [6]. However, there is a large consensus that dysbiosis and inflammation act according to a reciprocal cause-and-effect relationship, in which the microbiome induces inflammation and inflammation induces the selective growth of specific bacterial species [7].

Normally, the oral cavity is colonized by different types of bacteria, which may accumulate on dental surfaces in the form of biofilm before and plaque after, thus triggering the host's innate immune response to establish an inflammatory state that can result, over time, in chronic inflammation and in the pathogenesis of periodontal diseases, with progressive destruction of the teeth-supporting tissues [8,9].

According to this model of periodontal disease pathogenesis, the Gram-negative anaerobic bacterium *Porphyromonas gingivalis* is considered the keystone pathogen of periodontal disease [10], given that it has a pivotal role in initiating dysbiosis [11] and in progressing the disease [12]. In particular, *P. gingivalis* resides in the sub-gingival plaque and interacts with different bacterial species, such as some of the genus *Streptococcus* spp. [13], from which it benefits in terms of growth and resistance [14]. *Streptococcus mutans* is an early colonizer found in particular in the supra-gingival plaque that determines the formation of an acidic oral environment responsible for the development of dental caries [15]. Although evidence shows that *S. mutans* does not directly interact with *P. gingivalis* [16], the former is found at high levels in the sub-gingival plaque of patients with severe periodontitis, thus representing a high-risk factor for dental root caries development [17–21].

Currently, the first line of therapy for the treatment of periodontitis is the mechanical removal of plaque, the use of antimicrobial agents, and, in severe cases, the use of bone grafts to fill and regenerate the alveolar bone [22]. Grafting materials can be of several types, of natural or synthetic origin, and can be used alone or combined with antimicrobials or biological molecules [23], with the aim of providing structural support for teeth and dental implants and a bioactive action on the surrounding supportive tissues. In this context, plant-derived polyphenols have been shown to positively act on bone regeneration, thanks to their multitude of biological effects, ranging from anti-oxidant to anti-bacterial and anti-inflammatory [24]. Thanks to their ability to interplay with different molecular pathways involved in the promotion of osteogenesis and bone protection [25], their particular properties make them ideal candidates for the treatment of diseases characterized by dysregulation of inflammation and bacterial-induced damage.

In this work, we show the *in vitro* anti-bacterial and anti-inflammatory action of the Synergoss Red (SR) ceramic bone filler [26], coated with a polyphenol-rich grape pomace extract for which we previously demonstrated the ability to induce osteogenic differentiation in mesenchymal stem cells [25]. In addition to their well-known regenerative properties [27], phenolic compounds from grapes are of interest because they show a high-value potential for their versatile use in circular economy applications. In fact, a consistent amount of these molecules can be recovered from winemaking wastes, which account for about 20% of the starting material [28], thus giving the grape pomace a further valorization chance.

2. Materials and Methods

2.1. Materials

All chemicals were analytical-reagent grade. Ultra-pure (MilliQ) water was used for the preparation of aqueous solutions. All chemicals, namely acetone, acetic acid, Folin–Ciocâlteu reagent, 2,2-Diphenyl-1-picrylhydrazyl, sodium carbonate, beta-tricalcium phosphate gallic acid, quercetin 3-glucuronide, quercetin, tannic acid, catechin, malvidin 3-glucoside, epicatechin, procyanidin B2, myricetin, quercitrin, kaempferol, isorhamnetin, rutin, epigallocatechin gallate (EPGCG), caffeic acid, and trans p-cumaroyl tartaric acid were purchased from Sigma-Aldrich (St. Louis, MI, USA); hydroxyapatite was purchased from Fluidinova (Mala, Portugal); and red grape pomace was purchased from a local wine producer (Croatina grape from ALEMAT, Penango, Italy).

2.2. Synthesis of the Ceramic Bone Filler

Hydroxyapatite (HA) was used for enhancing the mechanical strength of scaffolds, whereas β -tricalcium phosphate (β -TCP) was used for its degradability; they were mixed in a percentage of 70/30 wt.%, respectively, to reach an optimal compromise between the

two properties. The ceramic scaffolds were prepared by mixing HA and β -TCP powders (47 wt.%) with the binding agent poly (vinyl alcohol) (3 wt.%) and ultrapure water (50 wt.%) to obtain a ceramic slurry. Dolapix CE 64 was added as a dispersing agent (1 wt.% of the solid load). The polyurethane (PU) sponge impregnation method was used to obtain a macroporous ceramic scaffold [29,30]. A commercial polyurethane (PU) sponge slab (45 ppi) of $200 \times 200 \times 10 \text{ mm}^3$ was soaked into the ceramic slurry for 90 s, followed by compression along the transverse plane (40 kPa), and left at room temperature for 5 min before repeating the cycle. Impregnation/compression cycles were repeated 3 times. The ceramic-coated PU sponge was left to dry overnight at 37°C and sintered in a furnace at 1100°C for 12 h in air (heating rate $5^\circ\text{C}/\text{min}$) to obtain a porous HA/ β TCP slab of $200 \times 200 \times 10 \text{ mm}^3$ (a volumetric retention of 24% was calculated). The obtained porous slab was ground in a jaw crusher (BB 50, Retsch GmbH, Haan, Germany) and sieved to obtain a 300–2000 μm porous-grained granulate (bone filler control) (BF).

2.3. Preparation of the Polyphenol-Rich Pomace Extracts (PRPE)

Grape pomace was received in dry form from the producer and stored at -20°C under vacuum until the beginning of the extraction process. In order to make it suitable for the extraction process, the grape pomace was first washed with acidified water, dried in a circulating-air oven ($37^\circ\text{C} \pm 5^\circ\text{C}$), and ground in a bladed mill (GM 200, Retsch). The milled grape pomace (300 g) was extracted in 2000 mL of 50:50 acetone–water (*v/v*) using an automatic extractor (Micro C TIMATIC, Spello (Pg), Italy). The extraction cycle is fully automatic and alternates a dynamic phase, performed at a programmed pressure and a static phase, in which a forced percolation is generated, which, thanks to the programmable recirculation, ensures a continuous flow of solvent to the interior of the plant matrix, thus avoiding over-saturation [26]. Next, the extracted solution was concentrated under reduced pressure in a rotavap in such a way to eliminate the acetone and to obtain an aqueous extract. The concentrated extract was separated by centrifugation (7000 rpm, 5 min), and the supernatant fraction was used to treat the ceramic granulates. High Performance Liquid Chromatography (HPLC) analysis was conducted on the extract, results are reported in Supplementary Materials.

2.4. Coating of Ceramic Bone Filler with PRPE

Synergoss Red was prepared by soaking the porous ceramic granulate with the polyphenol-rich pomace extract in a ratio of 40:60 wt.%, respectively, and left to evaporate for 24 h at 37°C to obtain a final ceramic bone filler enriched with polyphenols molecules.

2.5. Morphology Characterization of the Ceramic Granules

The surface morphology of the granules was analyzed through scanning electron microscopy (SEM) analysis. Samples were mounted on aluminum stubs and sputtered with gold at 15 mA for 2 min using Agar Sputter Coater. The morphology of the bone filler was captured using the EVO MA 10 system (Zeiss, 73447 Oberkochen, Germany).

Specific surface areas of the bone filler particles were measured by Kr adsorption–desorption isotherms at 77 K using a Micromeritics ASAP 2020 instrument (Micromeritics, Norcross, GA 30093, USA), and SSA was calculated by the Brunauer–Emmett–Teller (BET) method.

The microstructure of the granulate bone filler was analyzed by using micro-computed tomography (μ -CT), equipped with a desktop μ -CT scanner (SkyScan 1174, Aartselaar, Belgium), set at a voltage of 50 kV and a current of 800 μA , and the sample was scanned at a 9.23 μm pixel resolution. The exposure time per projection was 10,000 ms and a 720 μm aluminum filter was used. A total of 0.05 g of material was used as the sample. The CT-Analyzer software (version 5.30) was used for the imaging analysis.

2.6. Phase Analysis of the Ceramic Granules

The Analytical X'Pert Pro diffractometer (Malvern Panalytical, Malvern, Worcestershire, UK) equipped with an X'Celerator detector powder using Cu K α radiation generated at 45 kV and 40 mA, was used to characterize the crystal structure of the biphasic calcium phosphate bone filler. The samples were measured in the 2-theta, ranging from 20° to 40° at a rate of 0.2°/min.

2.7. Total Phenolic Content of PRPE

The initial phenolic content of Croatia PRPE was evaluated by using the Folin–Ciocâlteu (FC) method as previously reported [31–33]. The extract was transferred in a 25 mL volumetric flask and diluted 1:50 with distilled water (3 technical replicates). Then, 0.5 g of Folin–Ciocâlteu reagent was added and mixed for 5 min, and 1.5 g of 20% anhydrous sodium carbonate (*w/v*) solution was added. After 2 h, absorbance was measured at 765 nm by using water as the compensation liquid and a quartz cell (10 mm path length) in a UV–visible spectrophotometer (PG Instruments Limited, Lutterworth, UK). The absorbance value was used to calculate the concentration of polyphenols by using a calibration curve obtained with gallic acid. The results are expressed as mg/mL of gallic acid equivalents (GAE).

2.8. Calibration Curve

Ten milligrams of gallic acid were diluted in 10 mL of water to obtain 1 mg/mL of stock solution. Aliquots of stock solution were transferred in a 25 mL volumetric flask and diluted in water to the final concentrations of 0.05 mg/mL, 0.025 mg/mL, 0.01 mg/mL, and 0.005 mg/mL. Each standard solution was prepared according to the procedure described above for the PRPE; the absorbance was measured under the same conditions as for PRPE.

2.9. Antioxidant Power of PRPE

The antioxidant power of PRPE (1 mg/mL of GAE) and of Pph released from Synergoss Red was evaluated through the widely used DPPH (2,2-Diphenyl-1-picrylhydrazyl) method [34]. This test measures, through a colorimetric approach, the ability of the test solution to scavenge the DPPH radicals. Briefly, an aliquot of 40 μ L of PRPE was added to 1600 μ L of water–ethanol 50:50 (*v/v*) solution (3 technical replicates). Separately, a DPPH solution (0.1 mg/mL *w/v*) in ethanol was prepared and 2 mL of this solution was added to the reaction mixture. Then, the solution was shaken and incubated for 30 min at room temperature in the dark; the absorbance was recorded at 525 nm. The blank solution consisted of a solution of water–ethanol instead of PRPE. The inhibition percentage of the DPPH radicals by the samples was calculated using the following equation:

$$\% \text{ Reduction} = \left(\frac{A_0 - A_1}{A_0} \right) \times 100 \quad (1)$$

where A_0 is the absorbance of the control sample and A_1 is the absorbance of the test sample.

2.10. Polyphenols Release Study

The release study was performed on 0.2 g of material placed in a 12-well plate, filled with 1 mL of ultrapure water, for 24 h and 48 h at 37 °C. The amount of polyphenols released has been quantified by the Folin–Ciocâlteu method.

2.11. Cell Culture

The cell culture was set up as already described in a previous work [26]. Specifically, the murine macrophage cell line J774a.1, obtained from the Department of Cell Substrates at the Istituto Zooprofilattico Sperimentale of Lombardia and Emilia Romagna “Bruno Ubertini”, was used to evaluate the inflammatory response to the bone filler and the SR materials, both incubated in direct contact with cells. Briefly, cells were maintained in Dul-

becco's modified eagle medium (Gibco Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum, penicillin (100 U mL^{-1}), streptomycin ($100 \mu\text{g mL}^{-1}$), and 4 mM l-glutamine and left grow in a 100% humidified incubator at 37°C with 10% CO_2 and then passaged 2–3 days before use. Two different experiments were conducted with the same bone filler and SR materials: the first was before materials sterilization and the second was after sterilization with gamma rays at 25 kGy; for both experiments, the J774a.1 cells were seeded ($1.1 \times 10^6 \text{ mL}^{-1}$ for the unsterilized materials and $8.5 \times 10^4 \text{ mL}^{-1}$ for the sterilized materials) onto 12-well tissue culture polystyrene plates (Falcon® Corning™, Corning, NY, USA), containing the bone filler and the SR materials both at a concentration of 0.2 g/well.

2.12. Gene Expression Analysis by Real-Time Quantitative Reverse Transcription PCR (RT-qPCR)

Gene expression analysis was performed by following the same steps described in a previous article [26]. After 4 h incubation, the RNA from J774.a1 cells were isolated by using the Maxwell® RSC simplyRNA Cells Kit (Promega Corporation, Madison, WI, USA), following the manufacturer's instructions. RNA quantitation was performed using the Quantifluor RNA system kit in the Quantus Fluorometer (both from Promega Corporation), and the obtained total RNA was reverse-transcribed using a HighCapacity cDNA Reverse Transcription Kit in the Thermal Cycler 2720 (both from Thermo Fisher Scientific, Waltham, MA, USA) at the following conditions: 10 min at 25°C , 120 min at 37°C , and 5 min at 85°C , and maintained at 4°C until further experimentation. RT-qPCR was performed following the fast running protocol of the TaqMan® FastAdvanced Master Mix (Thermo Fisher Scientific, Inc.) in the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific, Inc.) using designed murine TaqMan® assays (Thermo Fisher Scientific, Inc.) to quantify gene expression of the following genes: interleukin-1 β (IL-1 β ; ID: Mm01336189_m1), interleukin-6 (IL-6; ID: Mm99999064_m1), tumor necrosis factor alpha (TNF- α ; ID: Mm00443258_m1), interleukin 10 (IL-10; ID: Mm99999062_m1), beclin 1 (BECN1; ID: Mm01265461_m1), microtubule-associated proteins 1A/1B light chain 3B (MAP1LC3b; ID: Mm00782868_sH), glyceraldehyde 3-phosphate dehydrogenase (GAPDH; ID: Mm99999915_g1), and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein ζ (YWHAZ; ID: Mm03950126_s1). The real-time PCR was performed in technical duplicate for all samples and targets in a total volume of 25 μL and the amplification was performed as follows: hold at 50°C for 2 min, hold at 95°C for 2 min, 40 cycles at 95°C for 1 s and 60°C for 20 s. All transcripts were normalized to the mean of the reference genes YWHAZ and GAPDH. The ΔCT values were calculated as $\text{CT}(\text{reference gene}) - \text{CT}(\text{gene of interest})$. $\Delta\Delta\text{Ct}$ values represent the mean difference of ΔCt between treated and control samples (polystyrene), thus higher $\Delta\Delta\text{Ct}$ values reflect induction of gene expression due to the treatment. Results are expressed as Mean $\pm$ 95% CI of 3 biological samples ($n = 3$).

2.13. Antimicrobial Study to Define the Minimal Inhibitory Concentration (MIC)

The MIC is the highest dilution (or the lowest concentration) of an antimicrobial agent that inhibits the growth of the test microorganism after 24 h under a standardized set of conditions. On the test items, a study was performed to define the minimal inhibitory concentration (MIC) against A90 *Streptococcus mutans* DSM20523 and B160 *Porphyromonas gingivalis* DSM207009. MIC against *S. mutans* was performed by broth dilution method in tubes and verified by streaking on a plate; MIC against *P. gingivalis* was performed by microbroth dilution method. The test item was a polyphenol-rich extract used to functionalize the Synergoss Red Bone filler, with an initial concentration of 3.2 mg/mL. As reference control, a solution of gallic acid, at the same concentration, was used. The MIC test was performed twice, in three independent replicates. Three series of not less than twelve dilutions of the test item and the reference item, respectively, were prepared and tested.

Dilution Method in Tubes: Tubes containing 1 mL of appropriate dilution to be tested were prepared. Series (1), (2), (4), and (5) were inoculated 1:1 with the test suspension at about $1\text{--}2 \times 10^6$ cfu/mL in order to obtain a final concentration of about 5×10^5 cfu/mL. The tubes of series (3) and (6) were not inoculated and were used as microbiological control. Moreover, 3 tubes of uninoculated Muller–Hinton broth calcium adjusted (MHBCA) were used as negative controls and 3 tubes containing MHBA inoculated 1:1 with the test suspension were used as positive controls. All tubes were incubated at $37 \text{ }^\circ\text{C} \pm 1 \text{ }^\circ\text{C}$ for 24 h.

Microbroth Dilution Method (in microplate): One 96-well Microplate containing 0.1 mL/well of appropriate dilution to be tested was prepared. Series (1), (2), (4), and (5) were inoculated 1:1 with the test suspension at about $1\text{--}2 \times 10^6$ cfu/mL in order to obtain a final concentration of about 5×10^5 cfu/mL. Wells of series (3) and (6) were not inoculated and were used as microbiological control. Moreover, 3 wells of uninoculated MHBCA were used as negative controls and 3 wells containing MHBCA inoculated 1:1 with the test suspension were used as positive controls. The 96-well microplate was incubated at $37 \pm 1 \text{ }^\circ\text{C}$ for 48 h under anaerobic conditions.

After incubation for optimal growing conditions (temperature/time and tubes/wells) were observed for the growth of the test organism (i.e., obvious button or definite turbidity), and results were recorded.

For the broth dilution method against *Streptococcus mutans* DSM20523, due to the evident turbidity of the test item dilutions in the tube, the MIC was also verified by streaking on TSA plates. For the microbroth dilution method against *Porphyromonas gingivalis* DSM20709, no interfering turbidity of the test item dilutions was observed. Verifying by streak on Columbia Agar plates was not necessary.

The growth was recorded with a (+) when it occurred, whereas a (−) was assigned when no growth was observed. Finally, the MIC value was determined.

The viable microorganism concentration in the inoculum was calculated by applying the following formula:

$$N = \frac{c}{(n_1 + 0.1n_2)d} \quad (2)$$

where c is the sum of colonies counted on all countable plates; n_1 is the number of counted plates in the lower dilution; n_2 is the number of counted plates in the higher dilution and d is the dilution factor corresponding to the lower dilution. The counting was performed using the number of colonies counted on Petri plates. The highest dilution (or the lowest concentration) of antimicrobial agent that inhibits the growth of the test microorganism after 24 h under a standardized set of conditions is considered the MIC value.

2.14. Statistical Analysis

Statistical analysis was performed using both the Analysis Tool pack in Excel, the PAST software [35] (Release 4.13), and the Real Statistics Resource Pack [36] (Release 5.11) on $\Delta\Delta\text{Cq}$ data of the different group treatments.

Data are represented as Mean $\pm$ 95% CI and include 3 biological replicates per treatment.

Before testing for differences between groups of means, we first assessed the independence of each other of all drawn samples, any deviation from the assumption of normality, by the Shapiro–Wilck statistical test, and any deviation from the assumption of equal variances, by the Levène statistical test.

In the case of all the above assumptions were met, one-way analysis of variance (one-way ANOVA) was performed, followed by the Tukey HSD post hoc test.

For TNF- α data from the experiment before sterilization, the quadratic transformation was performed to meet the assumption of normality.

In the case of unequal variances (as with TNF- α data from the experiment before sterilization and IL-1 β data from the experiment after sterilization), the Welch's ANOVA test was performed, followed by the Games–Howell post hoc test.

Transformed data, along with their CI, were backtransformed to their original scale before being reported in the graph.

Results with $p < 0.05$ (* = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$ and **** = $p < 0.0001$) are considered statistically significant.

3. Results

3.1. Ceramic Granules Characterization

Surface morphology has a relevant role in the interaction of the biological environment with biomaterial surfaces. The ceramic bone filler was made through a synthesis process that promotes the formation of a rough surface.

Figure 1a shows SEM images of the surface of granules of ceramic bone filler, at different magnifications ($50\times$, $500\times$, $5000\times$, and $7500\times$).

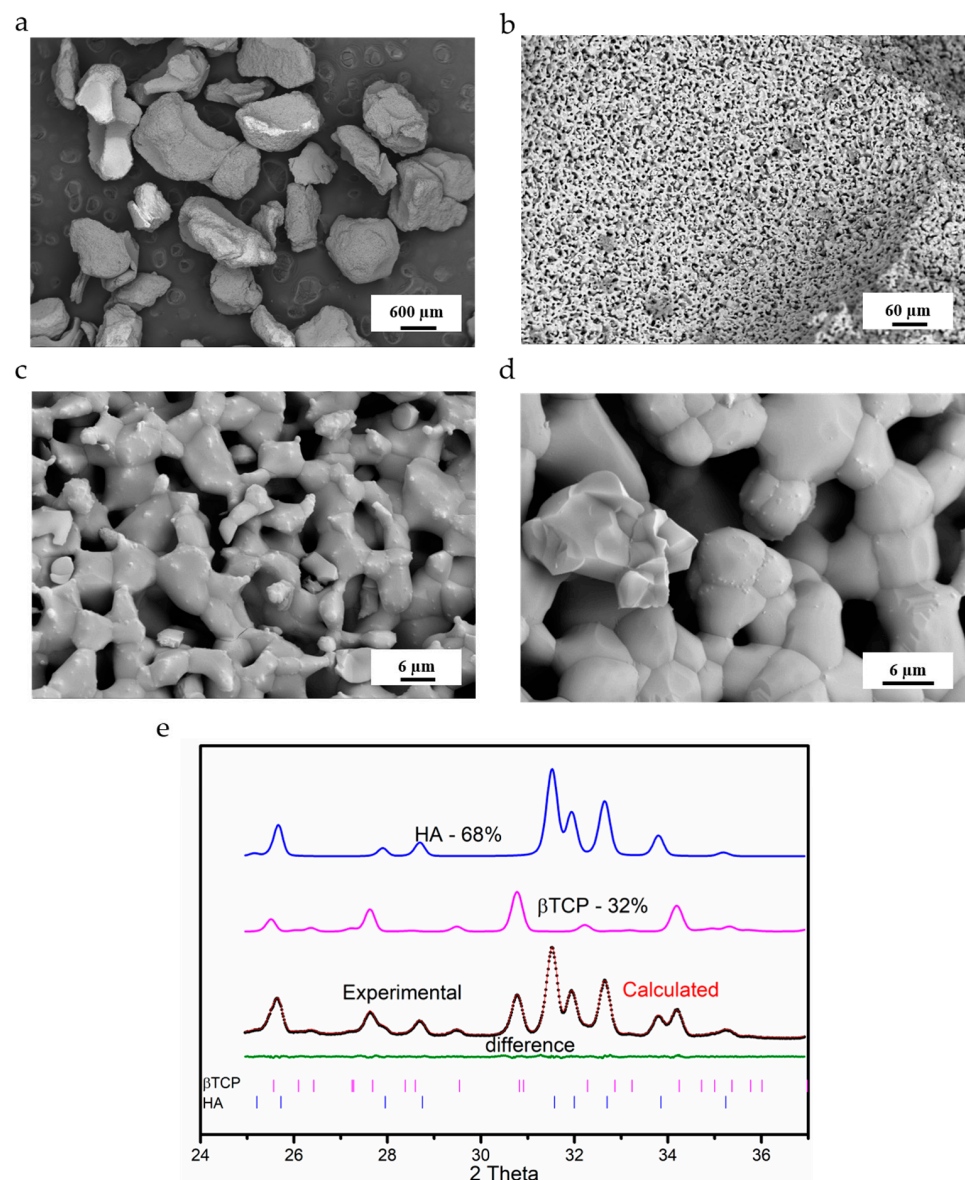


Figure 1. Images of the morphological structure obtained by scanning electron microscopy of the surface of ceramic bone filler at different magnifications (from upper left $50\times$ (a), $500\times$ (b), $5000\times$ (c), and $7500\times$ (d)). (e) XRD pattern of the sample biphasic bone filler (black dotted line), model patterns of hydroxyapatite (blue line) and β TCP (pink line), experimental data (red line) and the difference between the experimental profile and simulated one (green line). Peak positions for model HA and β TCP are pink and blue tick, respectively.

As it is possible to see in Figure 1a, granules were characterized by a diffused microporosity that influenced the surface area, which was measured using BET analysis and resulted in $1.64 \pm 0.06 \text{ m}^2/\text{g}$. The surface area also influences the ability of the material to uptake the polyphenol molecules. Different sintering, time, and ratios between HA and β TCP were tested, and the one that showed a higher polyphenols uptake (measured through the release test) and sufficient mechanical strength was selected (data of the optimization process can be provided under request).

Figure 1b represents the XRD pattern of the ceramic bone filler compared with the HA pattern, the β TCP pattern, and the results of a simulation of experimental data. As it is possible to see, the difference between the experimental profile and the simulated one is almost flat, thus meaning that the experimental X-ray pattern revealed the mass phase content of HA and β TCP to be 68% and 32%, respectively, considering the theoretical one of 70–30%.

The porosity, the pore size, and the open porosity were optimized and analyzed using the Micro-CT analysis. The porosity range is between 49.6 and 50.2%, and the pore size is between 100 and 1100 μm , with an open porosity percentage of around 98%. These parameters were obtained by optimizing the production process and the raw material ratio, allowing maximization of the bone filler loading capacity, and keeping the structural resistance and filling ability of the ceramic granulate.

3.2. Polyphenols Release Study from SR Bone Filler

The extract from Croatia grape pomace was analyzed by the Folin–Ciocâlteu method to evaluate the total phenolic content and showed an initial amount of 3.2 mg/mL of gallic acid equivalent (GAE) and a free radical scavenging activity of 80.5%. After functionalization of the ceramic bone filler with the polyphenol-rich extract, an in vitro release study was conducted at 24 h and 48 h. In Table 1 the values of phenolic content, expressed as mg/mL of GAE, and the antioxidant power, expressed as percentage of reduction, are reported.

Table 1. Total phenolic content (GAE mg/mL) and antioxidant power data reported as reduction percentage after 24 h and 48 h of release in ultrapure water.

Time	Total Phenolic Content Released (GAE mg/mL)	Reduction Power of the Released Solution (%)
24 h	0.95	18.3
48 h	1.06	19.6

3.3. Effects of SR on Expression of Genes Involved in Inflammation and Autophagy

The influence of the SR material on the expression of genes involved in inflammation and autophagy was evaluated both prior to and after sterilization. Results from the experiment involving unsterilized materials showed reduced expressions of IL-1 β and IL-6 induced by SR, compared to cells in contact with the ceramic bone filler (Figure 2A,B). In contrast, a higher TNF- α expression resulted in cells incubated with SR, compared to both the reference sample polystyrene and the bone filler (Figure 1C). A higher expression of IL-10 was also observed in cells in contact with SR, as compared to polystyrene, whereas the bone filler induced downregulation (Figure 2D).

Expression of BECN1 and MAP1LC3b genes was induced only in cells incubated with SR (Figure 3A,B).

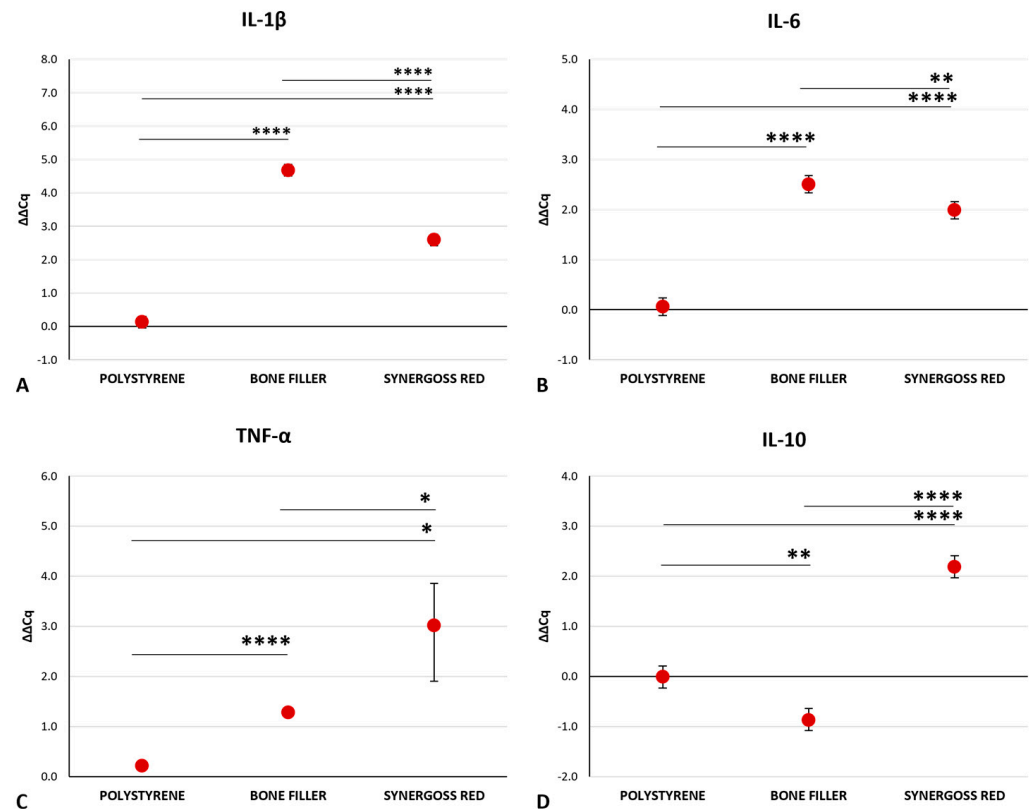


Figure 2. SR shows anti-inflammatory properties before sterilization. Quantitative real-time PCR analysis of mRNA levels of: (A) the key-inflammatory mediators IL-1 β , IL-6 (B), and TNF- α (C) and (D) the anti-inflammatory cytokine IL-10. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

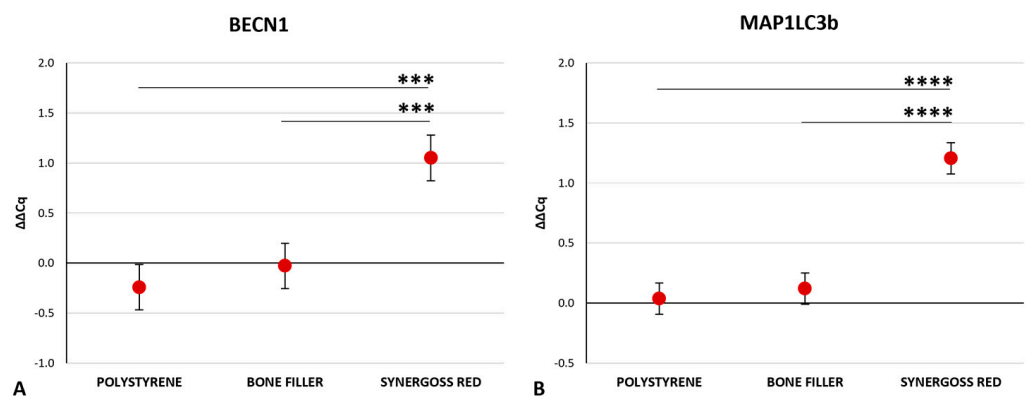


Figure 3. SR induces the expression of autophagy-related genes before sterilization. Quantitative real-time PCR analysis of the key-autophagy genes: (A) BECN1 and (B) MAP1LC3b. *** $p < 0.001$, **** $p < 0.0001$.

Interestingly, results from the experiment following sterilization (Figure 4A–C) showed reduced expression values for IL-1 β , IL-6, and TNF- α for both SR and bone filler, with the lowest levels found in cells in contact with SR.

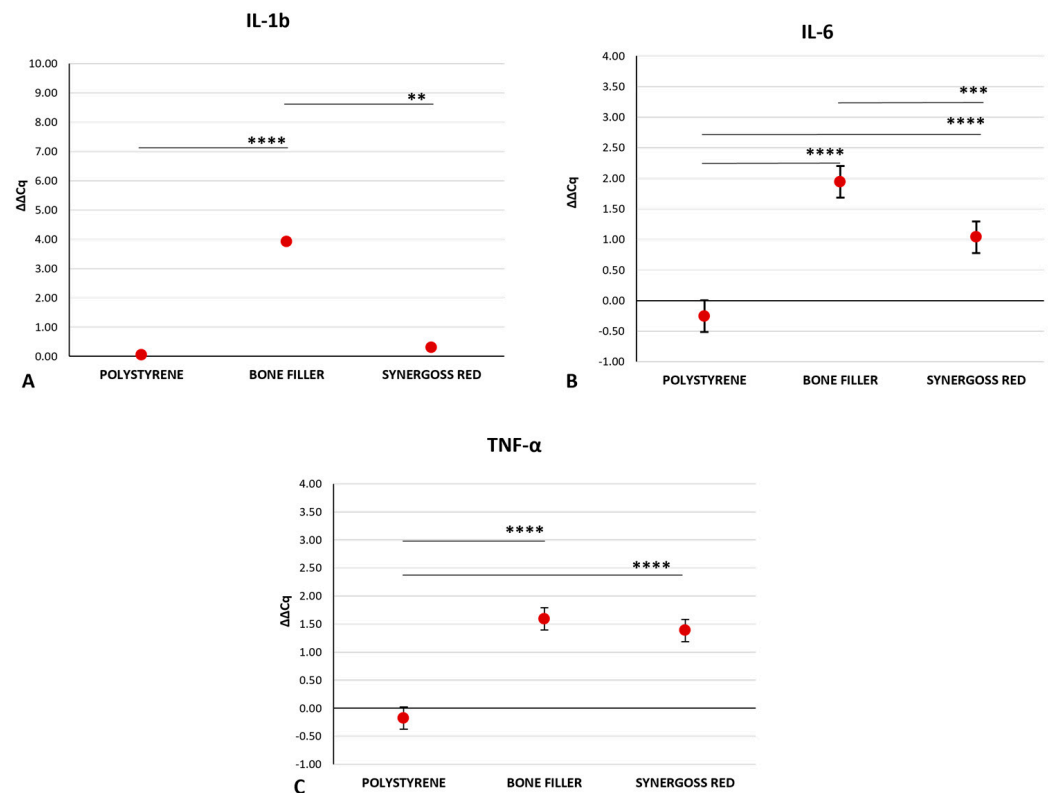


Figure 4. SR maintains its anti-inflammatory properties after sterilization. Quantitative real-time PCR analysis of mRNA levels of the key-inflammatory mediators: (A) IL-1 β , (B) IL-6, and (C) TNF- α . ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3.4. Antimicrobial Effect of the Polyphenol-Rich Extract

The results obtained by the antimicrobial study showed an average MIC of 0.8 mg/mL for the polyphenol-rich extract against *S. mutans*, and an average MIC of 0.8 mg/mL against *P. gingivalis*, whereas for the reference gallic acid standard solution, the MIC value cannot be defined against both microorganisms (Table 2).

Table 2. Minimal inhibitory concentration (MIC) of the polyphenol-rich extract and test control against *S. mutans* and *P. gingivalis*.

Organism	DSM No.	MIC (mg/mL)	
		Polyphenol-Rich Extract 3.2 mg/mL	Gallic Acid 3.2 mg/mL
<i>Streptococcus mutans</i>	20523, Type strain	0.8	-
<i>Porphyromonas gingivalis</i>	20709, Type strain	0.8	-

4. Discussion

Replacement of bone loss or restoration of bone defects is still a clinical challenge, especially because of disproportionate inflammatory reactions due to bacterial infections.

The mode of action of most of the commercially available bone fillers is to primarily provide tissues with mechanical support by acting as scaffolds for cell growth. Nowadays, the development of bone filler materials should be devoted to finding active biomolecules coupled with the ceramic material, which should demonstrate firstly biocompatibility (data showed in Supplementary Materials) and then could help in specific cases where the sole mechanical support is insufficient, such as periimplantitis.

Polyphenols represent a wide class of phytochemicals that are extensively studied for different purposes, ranging from medical to cosmetic, nutritional, and engineering applica-

tions, thanks to their numerous beneficial properties [37]. The medicinal properties of plant extracts have been known since ancient times, and their benefits also include important anti-bacterial activity against a large number of bacteria [38]. The polyphenol-rich extract used to functionalize our ceramic bone filler, SR, was shown to exhibit anti-bacterial activity against *S. mutans* and *P. gingivalis*. These results are impressive because they show that a natural extract can exert its anti-bacterial effects thanks to the synergistic actions of different phenolic molecules that compose the mixture; in fact, no specific anti-bacterial effects were shown for the reference “gallic acid” phenolic standard. Furthermore, the MIC value of the extract is comparable to the FC results obtained on the solution released by SR (Table 1), thus showing that the bone filler could exert an anti-bacterial effect following implantation. Although interesting and promising, these results need to be deeply investigated in further studies, in order to understand the precise mechanism of the anti-bacterial action of our extract. The ability of polyphenols to regulate inflammation in different biological systems is again well documented in several works [24]. Functionalization of our ceramic bone filler with the polyphenol-rich extract was intended to provide the oral tissues during surgical intervention with a ceramic bone filler with both anti-bacterial and anti-inflammatory actions to control bacterial growth that could drive disproportionate inflammatory reactions. As highlighted by the expression of genes involved in the early inflammatory response, SR determines the downregulation of the pro-inflammatory cytokines IL-1 β and IL-6, both prior to and especially after sterilization, as compared to the bone filler. These results demonstrate that the inflammatory cell response is induced by the material itself and that the polyphenol-rich extracts are responsible for the mitigation of this inflammation. These results are in line with those obtained in a previous study [26]. Furthermore, SR was shown to act on early inflammation induced by the material without, however, depleting its related transcripts, a key-factor aspect of the appropriate tissue repair process occurring after a wound or a surgical intervention [39,40]. Looking at TNF- α results both prior and after sterilization, no significant differences between the ceramic bone filler and SR can be appreciated, and this can be translated in a positive aspect; in fact, an early TNF- α signaling is specifically implicated in early fracture repair [41]. During bone repair, inflammation is involved in complex interplays with several biological mechanisms that tailor a sophisticated and controlled process. Among them, autophagy is a highly conserved cellular process implicated in different biological mechanisms, ranging from vesicular transport to energy control and cell death mediation [42]. In our study, expression of the two autophagic key genes BECN1 and MAP1LC3b was enhanced in cells at contact with SR; thus, this action of modulation of the autophagic process can be attributed to the polyphenolic extract itself. In this regard, autophagy has also been recognized as a process involved in immunomodulation; in fact, it directly regulates the production of inflammatory cytokines by inhibiting the inflammasome, with direct consequences on the production of IL-1 β , which is proteolytically degraded at the pro-IL-1 β stage and is therefore inhibited [43]. The influence of autophagy on cytokine expression also reflects on bone homeostasis; in fact, inhibition of autophagy in periodontal tissues resulted in increased expression of IL-1, IL-6, and TNF- α , leading to a reduced decline in bone density, while restoration of autophagy reduced, in particular, IL-6 expression [44]. It is also proven that autophagy is dysregulated in periodontitis [45] and that bone resorption occurs in cases of abnormal expression of cytokines; for example, overexpression of IL-6 following autophagy inhibition leads to reduced osteoprotegerin gene expression [46]. Furthermore, the ability of autophagy to influence the immune system is also shown by regulation of macrophage polarization. Specifically, induction of autophagy was shown to shift macrophage polarization to the M2 phenotype, the one involved in tissue repair, to attenuate inflammatory reactions [47], thanks to its anti-inflammatory activity and to the expression of the anti-inflammatory cytokine IL-10. Our results show that SR induced a higher expression of IL-10 as compared to the ceramic bone filler and, thus, that the phenolic extract is central to this action, as already shown in a previous work of ours [26]. Phenolic compounds can target different molecular pathways involved in several biological processes, such as energy homeostasis

and bone anabolism [24]. In this regard, different polyphenols were shown to regulate the autophagic process, with consequent immunomodulatory effects, among others [48]. In fact, thanks to their antioxidant power, polyphenols can modulate the redox signaling mediated by ROS/RNS, which can give rise to both reversible and irreversible modifications, leading the cell to carry out protective mechanisms, among which autophagy is a pro-survival process. In particular, oxidative stress is responsible for the concomitant induction of autophagy and the antioxidant machinery to decrease oxidative damage and restore cell homeostasis [49]. Autophagy is induced in response to cell damage or other physiological stimuli aimed at restoring and preserving cell homeostasis by promoting the removal of debris and damaged cellular components and, as such, plays a crucial role in the degradation of bacteria and their products. Although it was demonstrated that *P. gingivalis* infection takes advantage of the inactivation of the mTOR pathway in oral epithelial cells [50], activation of autophagy provides an effective defensive response against bacterial infections [51,52] and regulates innate immunity [53]. In this regard, the ability of the ceramic bone filler SR, functionalized with a polyphenol-rich extract, to downregulate inflammation, increase autophagic gene expression, and contrast bacterial growth provides a promising tool for fighting periodontal disease.

5. Conclusions

In the context of periodontal disease, in which the organism is constantly subject to the consequences of a dysbiotic microenvironment, there is a need for innovative applications designed to face the multiple challenges that this pathologic condition poses. The results presented in this work are promising in that they provide the basis for the development of new concepts and strategies to design bone fillers that provide both support by acting as scaffolds for cells and, at the same time, the control of bacterial growth and inflammation thanks to the regenerative properties of wine polyphenols extracted from byproducts.

Further experiments are planned to confirm and clarify the involvement of autophagy in polyphenol action, in addition to evaluating their potential for inhibiting biofilm formation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ceramics7030063/s1>, Figure S1: HPLC analysis of grape pomace extract; Table S1: Details of the HPLC gradient method used for chromatographic analysis; Table S2: Polyphenolic quantification through HPLC-DAD analysis; Figure S2: Cell viability assay of L929 fibroblast cells incubated in contact with Synergoss Red bone filler.

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