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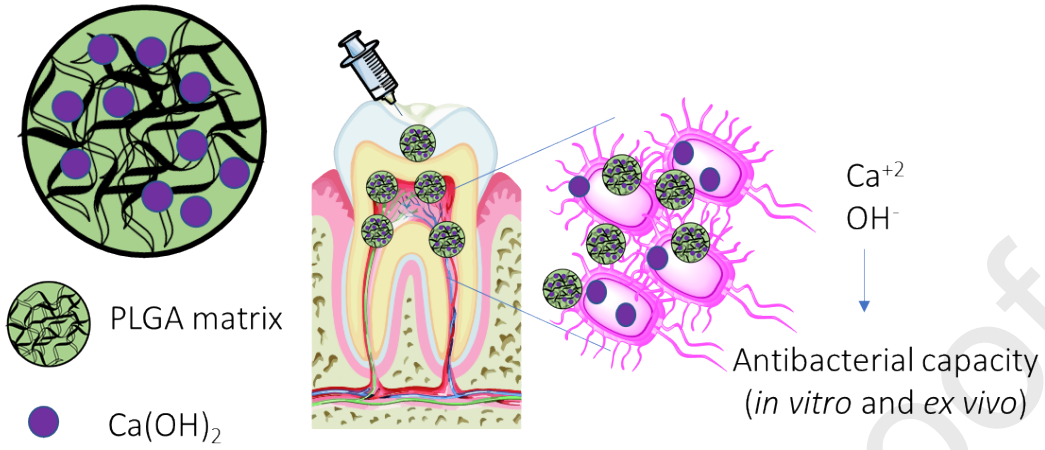
**Graphical abstract**



# Novel strategies enhancing endodontic disinfection: antibacterial biodegradable calcium hydroxide nanoparticles in an ex vivo model

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**Declaration of competing interest**

Dr. Firas Elmsmari, Dr. Fernando Duran-Sindreu, Dr. Marisa García López Dr. Jose Antonio González Sánchez and Dr Elena Sánchez López, report a licensed patent with the Universitat Internacional De Catalunya and University of Barcelona—Composition comprising nanoparticles, method for the preparation of a composition comprising nanoparticles and uses of the composition for dental treatment. European patent with the file number: EP20382504. The other authors have stated explicitly that there are no conflicts of interest in connection with this article.

All authors have reviewed the contents of the manuscript being submitted, approved its contents, and validated the accuracy of the data.

**Abstract:** Due to the high failure rates associated to endodontic disinfection, this study aimed to investigate the antibacterial properties of poly(lactic-co-glycolic acid) (PLGA) nanoparticles (NPs) loaded with $Ca(OH)_2$ for endodontic disinfection procedures. $Ca(OH)_2$ NPs production and physicochemical characterization were carried out as well as multiple antibacterial tests using three bacterial strains and an ex vivo model of endodontic infection with extracted human teeth. Agar diffusion test and broth dilution determined the inhibition growth zones (n=5) and the minimal inhibitory concentration (MIC, n=5), respectively. Cell viability was assessed using Live/Dead staining with confocal microscopy (n=5). Data was analysed using ANOVA followed by post-hoc analysis. After 24 hours of incubation, $Ca(OH)_2$ NPs demonstrated a MIC of 10 µg/mL for *Porphyromonas gingivalis* ($p<0.001$) and *Enterococcus faecalis* and 5 µg/mL for *Fusobacterium nucleatum* ($p<0.001$). Although the agar diffusion test did not exhibit any inhibition for $Ca(OH)_2$ nor for $Ca(OH)_2$ NPs area probably due to the buffering effect of the agar medium. However, the antibacterial capacity was confirmed in an ex vivo model, where instrumentalized teeth were infected with Enterococcus Faecalis and treated after 28 days of culture. A significant reduction in bacterial metabolic activity was confirmed for $Ca(OH)_2$ NPs (40% reduction with a single dose) and confirmed by Live/Dead staining. In conclusion, $Ca(OH)_2$-loaded PLGA NPs present promising antibacterial efficacy for endodontic disinfection procedures.

**Keywords:** anti-bacterial agents; calcium hydroxide; disinfection; endodontics; enterococcus faecalis; nanoparticles; in vitro antibacterial model.

## 1. Introduction

The American Association of Endodontics (AAE) reports that over 15.1 million root canal therapy procedures are performed annually in the United States [1]. While success rates for this procedure can be as high as 95%, success rates decrease in cases diagnosed with necrotic non-vital pulp tissue, which is often caused by pathogenic microorganisms [2,3]. The root canal system possesses a complex architecture and the location of microorganisms in isthmuses, accessory and lateral canals, and dentinal tubules can make complete eradication of bacteria highly challenging [4–8]. Even with mechanical debridement and chemical cleaning, completely eliminating bacteria from the root canal system is difficult to achieve [9,10]. Intracanal medications are used between visits to decrease bacterial load, but their efficacy is limited, especially for bacteria residing in anatomically complex areas such as the dentinal tubules [11]. Despite advances in endodontics, success rates have remained similar over the decades, indicating that current intracanal medications have limitations and cannot achieve the desired effect [3,12].

Calcium hydroxide ($Ca(OH)_2$) is the most commonly used to supplement chemomechanical preparation to enhance disinfection within the root canal system [26]. Its antibacterial activity is due to its alkalinity, which produces highly oxidant hydroxyl ions that act on the bacterial cell wall, resulting in damage to the cytoplasmic membrane through protein denaturation as well as DNA damage [13]. In addition, a high basic pH must be maintained to sustain the hydroxyl ions antibacterial activity, which alters the pH gradient of the cytoplasmic membrane, leading to protein denaturation [14]. $Ca(OH)_2$ elevated pH also damages the organic components of the cytoplasmic membrane, inhibits nutrient delivery, and causes DNA strand splitting, ultimately leading to DNA replication inhibition and harmful mutations, disrupting cellular activity [15]. However, evidence suggests that all three mechanisms of damage to the bacterial cytoplasmic membrane, protein denaturation and DNA damage, may occur simultaneously, and it is challenging to establish a chronological order in which these events take place [15,16]. $Ca(OH)_2$ uncoupling into calcium and hydroxyl ions is highly dependent on the vehicle used for the application, affecting the pH value and the degree of penetration inside the tubules [17]. Therefore, a suitable vehicle should allow high penetration through the tubules and a slow and steady release of calcium and hydroxyl ions with no undesirable effects on the initiation of hard tissue formation. In terms of intracanal medications there is still research and patent work to be undertaken, specially regarding the precise dose, treatment duration, dispensing method and delivery vehicle [18]**.** Although different vehicles have been used to administer $Ca(OH)_2$, such as water-soluble, viscous, and oil-based vehicles [14], all of them carry a certain degree of adverse effects, ultimately affecting $Ca(OH)_2$ clinical performance [14]. Therefore, there is an urgent medical need for a suitable and safe vehicle able to maintain $Ca(OH)_2$ properties during a prolonged time.

Additionally, in endodontics, $Ca(OH)_2$ paste has also been utilized in the majority of regenerative endodontic procedures ahead of the triple antibiotic paste, according to a study conducted in 13 countries [19]. This may be because the triple antibiotic has unfavorable side effects like staining teeth. Therefore, in cases of regenerative endodontic procedure, the American Association of Endodontics and the European Endodontic Society currently advise using $Ca(OH)_2$ paste.

Over the years, mixed results have been obtained regarding the antibacterial effectiveness of $Ca(OH)_2$ in eliminating bacteria from the root canal system [20–23]. While

many studies reported the high efficacy of $Ca(OH)_2$ as an antibacterial agent, others documented its inefficacy in eradicating bacteria and their by-products, especially in conditions similar to the clinical environment [24–26]. Furthermore, $Ca(OH)_2$ has been shown to be ineffective in removing microorganisms that settle inside the dentinal tubules [11] because it cannot directly contact the bacteria inside the tubules, which is essential for exerting its antibacterial effects [27,28]. This is likely due to the limited penetration of $Ca(OH)_2$, which can only reach up to 28 and 126 µm inside the dentinal tubules, whereas bacteria can penetrate up to 400 µm in some circumstances [29,30].

To overcome these challenges without using new molecules that would require an extended follow-up at clinical level and improve the efficacy of $Ca(OH)_2$, recent drug delivery procedures have focused on nanotechnological approaches able to load active compounds and deliver them in a prolonged manner retaining the pharmaceutical properties [31–35]. Among several nanoscopic systems, biodegradable polymeric nanoparticles (NPs) have shown to possess suitable properties and, especially poly-(lactic-co-glycolic) acid (PLGA), is accepted by the main regulatory agencies [36–38]. PLGA NPs may be able to decrease potential adverse effects, and prevent or reduce the buffering effect produced by dentin and hydroxyapatite, as well as maintain the high alkaline pH value in which $Ca(OH)_2$ can retain its antibacterial capability [14,35]. In a previous study [39], our research team successfully optimized $Ca(OH)_2$-loaded PLGA NPs ($Ca(OH)_2$ NPs that displayed an extended drug release profile compared to free $Ca(OH)_2$ and significantly greater infiltration inside dentinal tubules of extracted teeth in contrast to free $Ca(OH)_2$. The current study aims to examine the antibacterial capacity of this optimized $Ca(OH)_2$ NPs against three bacterial strains in order to elucidate the suitability of $Ca(OH)_2$ NPs for endodontic disinfection. The null hypothesis was that there was no difference in the antibacterial activity between $Ca(OH)_2$, $Ca(OH)_2$ nanoparticles, and control group against endodontic bacteria.

## 2. Materials and methods

This study was conducted with the approval of the ethical committee with code (END-ELB-2020-01) to assess the antibacterial effect of $Ca(OH)_2$ NPs for endodontics disinfection procedures. The antibacterial efficacy of the NPs was evaluated through several antibacterial tests using three bacterial strains: *Porphyromonas gingivalis* (Pg) (ATCC 33277), *Fusobacterium nucleatum* (Fn) (ATCC 25586), and *Enterococcus faecalis* (Ef) (ATCC 19433) [40–42], in accordance with the guidelines proposed by the Clinical and Laboratory Standards Institute (CLSI) [43,44].

### *2.1 Preparation of calcium hydroxide nanoparticles*

The preparation and characterization of $Ca(OH)_2$ NPs has been carried out as described elsewhere [39]. Briefly, $Ca(OH)_2$ NPs were prepared using the solvent displacement method, and they were optimized using a central composite design. The optimized $Ca(OH)_2$ NPs were measured using photon correlation spectroscopy (PCS) to determine the average size and polydispersity index (PI) after 1:10 dilution at 25°C, using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). In addition, transmission electron microscope observation was

carried out after negative staining using uranyl acetate (2%) and measurement of the diameter of the $Ca(OH)_2$ NPs was carried out using ImageJ software [45–47]. Subsequently, the antibacterial capacity was assessed using several methods (Figure 1).

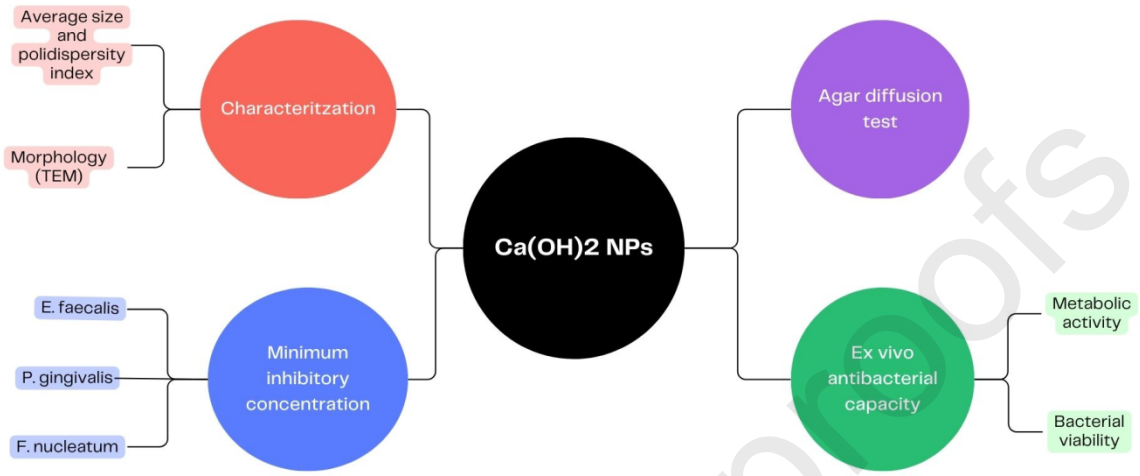


**Figure 1.** Flowchart of the experiments carried out in the present study.

### *2.2 Minimal inhibitory concentration (MIC)*

To determine the minimal inhibitory concentration (MIC), a broth microdilution method was used according to the guidelines proposed by the Clinical and Laboratory Standards Institute (CLSI) [43,44] (Figure 2A). Serial dilutions were carried out (1:2, 1:5, 1:10, 1:20, 1:50, 1:100, 1:200; 1:500) for both the $Ca(OH)_2$ nanoparticles (NPs) and Calcium hydroxide 98% extra pure ACROS Organics™ (Fisher Scientific, USA) mixed with Milli-Q water at the same concentrations, starting at a concentration of 1 mg/mL. Subsequently, 100 µl from each dilution was added to a 96-Well Microplate (Fisher Scientific, USA).

The bacterial suspension turbidity for the three bacterial strains *Porphyromonas gingivalis, Fusobacterium nucleatum,* and *Enterococcus faecalis* was adjusted to an optical density of 0.1 (equivalent to 0.5 McFarland) by adding brain heart infusion (BHI, Condalab, Spain) media and measuring absorbance in a spectrophotometer (Cary 60 UV-Vis Spectrophotometer, Agilent, USA) at a wavelength of 600 nm. Then, each diluted well was inoculated with 100 µl of the adjusted bacterial suspensions [48,49]. All experiments were performed by triplicate.

After 24 hours, bacterial growth was assessed in terms of turbidity, which was measured at 600 nm using a plate spectrophotometer (Infinite M Nano, TECAN, Switzerland). Wells containing the broth medium only were used as a negative control to examine the equipment and medium sterility. Additionally, some wells containing the broth growing medium and bacteria were used as a positive control to test the growing ability of the medium. The MIC was defined as the lowest concentration of the antimicrobial agent that inhibited bacterial growth [43], and the significance level was determined.

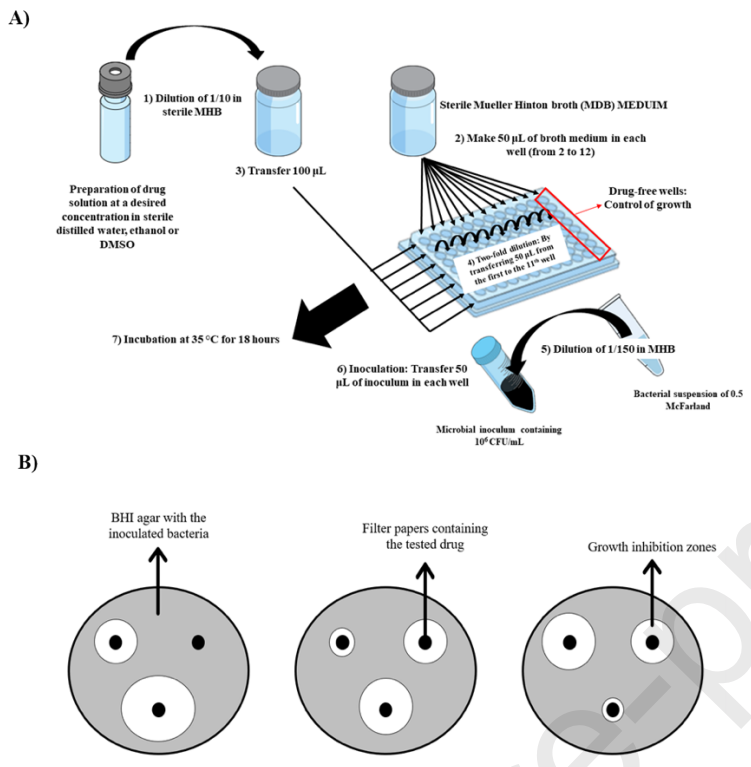


**Figure 2.** Scheme of the bacterial assays developed. A) Microdilutions used for antibacterial assesments as recommended by CLSI protocol and B) Agar diffusion test protocol.

### *2.3 In vitro antibacterial therapeutic efficacy*

To measure the growth inhibition zones, the agar diffusion test was used (n=3) [48,49]. Brain heart infusion agar (BHI Agar) (Condalab, Spain) plates were prepared and inoculated with *Enterococcus faecalis*, which has a high prevalence in cases of necrotic endodontic infections [50–52]. The antibacterial properties of $Ca(OH)_2$ NPs, free $Ca(OH)_2$, and a control (1x PBS) were tested as described elsewhere [44]. The plates were incubated under anaerobic conditions at 37°C for 24 hours, and the diameters of the inhibition growth were measured for each compound. All experiments were performed by triplicate (Figure 2B).

### *2.4 Ex vivo antibacterial therapeutic efficacy*

For the *ex vivo* model, extracted single-root teeth with straight canals were used, with the patient informed consent. No data associated with the patient was recorded concerning the extracted teeth. The extracted teeth were preserved in individual containers in a saline solution with 0.5% sodium hypochlorite (NaOCl, Proclinic, Spain) [39].

Root canal opening access was performed with a round diamond bur at high speed, and then the teeth were crowned, standardizing their length to 13 mm. Subsequently, root canals were instrumented with Reciproc Blue R25® (VDW, Germany) according to manufacturer instructions. 4.2% NaOCl irrigation was used during the instrumentation process to allow the correct progression of the instrument inside the root canal and to simulate clinical conditions. Then, teeth were cut horizontally with a diamond blade and a clinical handpiece at 3 and 6 mm from the apex, dividing the teeth into coronal, medial, and apical blocks. Finally, teeth were randomly divided into three experimental groups (control, $Ca(OH)_2$ NPs, and $Ca(OH)_2$) and were sterilized by gas plasma (Sterrad, ASP, USA).

Sterile samples were incubated in BHI media for two days to ensure complete rehydration and sterility. Then, they were inoculated with *Enterococcus faecalis* at an optical density of 0.1 at a wavelength of 600 nm. Samples were incubated under anaerobic conditions for 28 days with media renewal every two days to develop a mature biofilm [53]. Finally, treatments with $Ca(OH)_2$ NPs or $Ca(OH)_2$ at 1 mg/mL were applied.

### *2.5 In vitro metabolic activity*

Resazurin assay was used to quantify the metabolic activity, as it is proportional to the number of bacteria and their viability (n=5). The samples were washed twice with PBS and incubated with 300 µL of resazurin sodium salt at 30 µg/mL (Sigma-Aldrich, Spain) for 30 minutes at 37ºC. The absorbance was measured at 570 and 600 nm using 100 µL of each sample (Infinite M Nano, TECAN, Switzerland). The metabolic activity was normalized against the control, consisting of teeth incubated for 28 days without antibacterial treatment, and considered 100% metabolic activity.

### *2.6 Visual observation of bacterial viability*

In order to visually observe bacterial viability, it was assessed using Live/Dead staining with confocal microscopy observation. The samples were stained with LIVE/DEAD® BackLight™ Bacterial Viability Kit (Invitrogen™, Spain) according to the manufacturer's instructions. Adherent bacteria were stained with 300 µL of the dye-solution reagent for 15 minutes at 37ºC, and then washed with 1x PBS. Images were acquired at three random coronal, medial, and apical regions using a confocal laser microscope at 10x magnification (DMI8, Leica, Germany) using FITC and Texas Red excitation/emission filters for live and dead cells, respectively.

### *2.7 Statistical analysis*

All experiments were performed in duplicate by independent operators and the same supervisor. The data were analyzed using GraphPad Prism v6 (Graphpad software, Inc). The mean and standard deviation (±) were used to present the data. Significant differences were assessed by applying either one or two-way ANOVA followed by post-hoc analysis, with a significance threshold of 0.05.

## 3. Results and discussion

### *3.1 Average size of calcium hydroxide nanoparticles*

The optimized formulation of $Ca(OH)_2$ NPs was characterized by means of PCS obtaining a PI of 0.077 and an average size around 170 nm [39]. Moreover, surface charge was highly negative and NPs demonstrated the ability to encapsulate $Ca(OH)_2$. These results are in accordance with other formulations based on PLGA nanoparticles encapsulating different compounds [38,45,55]. Moreover, these data was supported by TEM nanoparticles observation where $Ca(OH)_2$ NPs were found to be spherical and non-aggregated (Figure 3A). Moreover, the average size of the obtained images was analyzed and frequency distribution was calculated (Figure 3B). An average size of 129 nm was calculated with all diameters being less than 200 nm, lower than the obtained using photon correlation spectroscopy. Moreover, frequency distribution obtained by both techniques show similar results with smaller nanoparticles in the case of TEM measurements (Figure 3C, 3D).

Since a single parameter can not be used to adequately describe sample distribution, less than 200 nm particle size and spherical shape was confirmed using PCS and TEM investigations [54]. Moreover, due to the measurement of the hydrodynamic ratio by PCS, TEM results provide slightly small nanoparticles since it constitutes a direct measurement [55]. Moreover, pH was also measured obtaining an alkaline pH (9.65) that favours calcium hydroxide therapeutic efficacy.

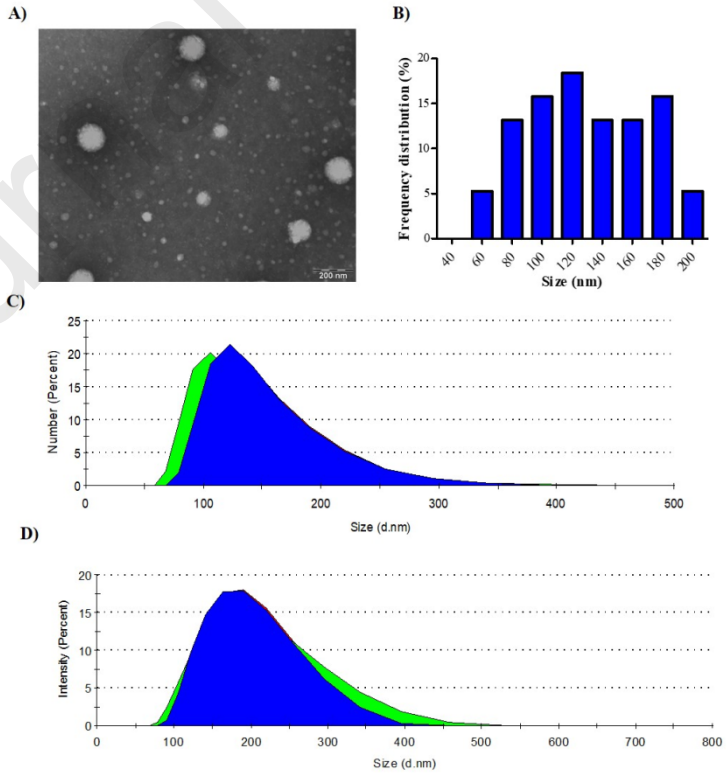

**Figure 3.** Morphological and physicochemical characterization of calcium hydroxide nanoparticles. A) Transmission electron microscope results, B) Frequency distribution results of the average size of transmission electron images obtained, C) Frequency distribution in number of particles obtained using dynamic light scattering, D) Frequency distribution in intensity obtained using dynamic light scattering.

### *3.2. Minimal Inhibitory Concentration (MIC)*

After 24 hours of incubation, the antibacterial activity of $Ca(OH)_2$ NPs and free $Ca(OH)_2$ was assessed against three bacterial strains, *Porphyromonas gingivalis, Fusobacterium nucleatum,* and *Enterococcus faecalis,* and was evaluated after several dilutions (Figure 3). As can be observed on Figure 4, both $Ca(OH)_2$ NPs and free $Ca(OH)_2$ showed statistical differences ($p<0.0001$) against the control thus highlighting their antibacterial activity. Furthermore, at low concentrations (2 µg/mL for E. faecalis *and F. nucleatum* and 2 and 5 µg/mL *for P. gingivalis)*, due to $Ca(OH)_2$ NPs prolonged release, statistical differences were obtained.

As can be observed in Table 1, $Ca(OH)_2$ NPs had a MIC of 10 µg/mL for *Porphyromonas gingivalis* and *Enterococcus faecalis* and 5 µg/mL for *Fusobacterium nucleatum*. $Ca(OH)_2$ had a MIC of 5 µg/mL for *Enterococcus faecalis* and was below 1 µg/mL for *Fusobacterium nucleatum* and *Porphyromonas gingivalis* (Table 1). Therefore, $Ca(OH)_2$ NPs showed the ability to retain $Ca(OH)_2$ antibacterial activity against different bacterial strains. Moreover, as previously demonstrated, $Ca(OH)_2$ NPs released the active compound in a prolonged manner showing higher MIC. The proposed $Ca(OH)_2$ NPs reduced the MIC concentration reported by previous studies against *Enterococcus faecalis* and *Porphyromonas gingivalis* [57] and, in addition, a previous attempt to produced $Ca(OH)_2$ NPs obtained a similar MIC reduction (6 µg/ml approximately). However, these NPs were not biodegradable [59].

In addition to the agar diffusion test, the minimum inhibitory concentration (MIC) of $Ca(OH)_2$ NPs was compared to commercial $Ca(OH)_2$ in terms of inhibiting bacterial growth for three bacterial species. It was noted that after 24 hours of incubation, both $Ca(OH)_2$ NPs and the commercial $Ca(OH)_2$ could inhibit bacterial growth at all tested concentrations [56,58,60–62]. Moreover, $Ca(OH)_2$ NPs MIC was higher than the commercial free $Ca(OH)_2$ thus confirming $Ca(OH)_2$ prolonged release from $Ca(OH)_2$ NPs.

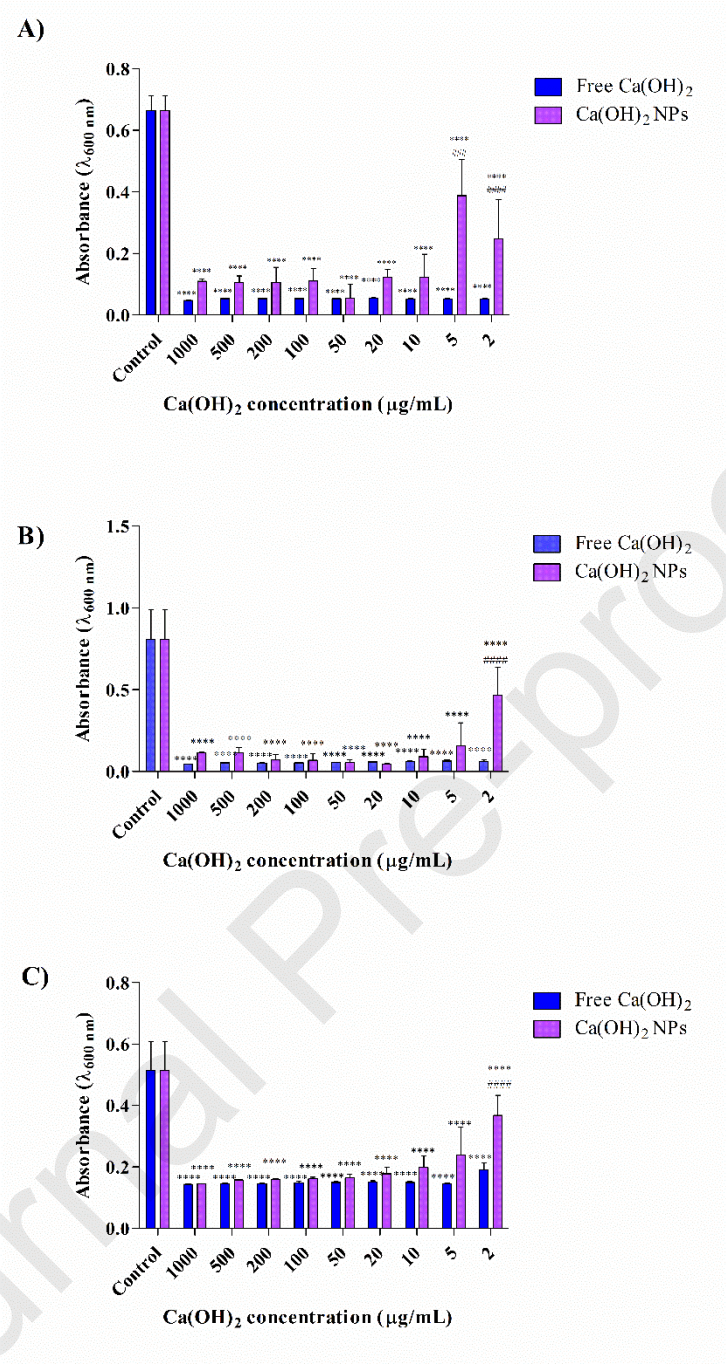


**Figure 4**. Absorbance obtained assessing the minimal inhibitory concentration (MIC) of three bacterial strains **A)** *Porphyromonas gingivalis*, **B)** *Fusobacterium nucleatum* and **C)** *Enterococcus faecalis* with different concentrations after 24 hours. Statistical significance was calculated using two-way ANOVA followed by Bonferroni post-hoc test. Significant differences against the control are represented as **** $p<0.0001$ and differences between the same concentration between free $Ca(OH)_2$ and $Ca(OH)_2$ NPs are represented as ### $p<0.001$ and ##### $p<0.0001$.

Table 1. Minimum inhibitory concentration values of Free $Ca(OH)_2$ and $Ca(OH)_2$NPs

| | Free $Ca(OH)_2$ | $Ca(OH)_2$NPs |
|---|---|---|
| *Porphyromonas gingivalis* | < 1 µg/mL | 10 µg/mL |
| *Fusobacterium nucleatum* | < 1 µg/mL | 5 µg/mL |
| *Enterococcus faecalis* | 5 µg/mL | 10 µg/mL |

### *3.2 Agar diffusion test*

The antibacterial performance of $Ca(OH)_2$ and $Ca(OH)_2$ NPs was also evaluated using the agar diffusion test against *Enterococcus faecalis* strain, which measures the growth inhibition zones in an agar plate. PBS was used as a control. However, using this assessment, neither $Ca(OH)_2$ nor $Ca(OH)_2$ NPs induced an observable inhibition area (Figure 5). Therefore, these results indicate that the active compound is not able to produce inhibition of the bacterial growth under the study conditions. This may be due to the fact that the agar diffusion test uses BHI agar media which possesses a neutral pH (pH 7.4 ± 0.2). Therefore, this assessment confirmed that either free $Ca(OH)_2$ or $Ca(OH)_2$ released from NPs both need a high pH environment in order to exert its effect due to the fact that the dissociation of calcium and hydroxyl radicals is necessary in order to obtain bacterial inhibition [63].

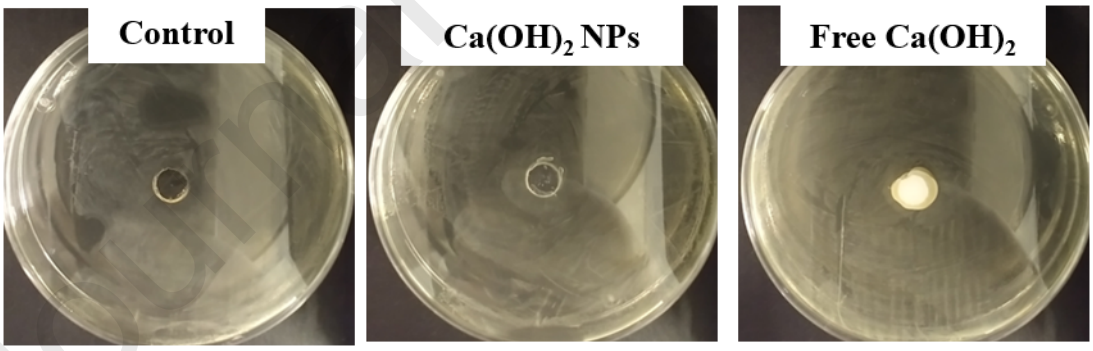


**Figure 5**. Agar diffusion test of $Ca(OH)_2$ NPs and calcium hydroxide at 1 mg/mL using *Enterococcus faecalis* strain at 24 hours. 1x PBS as control.

### *3.3 Ex vivo metabolic activity and bacterial viability*

The metabolic activity of bacteria infecting teeth and treated with either $Ca(OH)_2$ NPs or free $Ca(OH)_2$ was analysed using the resazurin reduction assay. The results demonstrated

that both $Ca(OH)_2$ NPs and $Ca(OH)_2$ significantly reduced ($p<0.05$) the metabolic activity compared to the control (Figure 6). Furthermore, although no significant differences between free $Ca(OH)_2$ and $Ca(OH)_2$ NPs were observed ($p>0.05$), $Ca(OH)_2$ NPs showed a trend towards a more marketed reduction of the metabolic bacterial activity. This may be due to the fact that the NPs are able to interact with the bacterial membranes in a more effective manner and also due to NPs prolonged release, $Ca(OH)_2$ protection against degradation and higher internalization in the dentinal tubules. Similarly, Podbielski et al. [65] showed that $Ca(OH)_2$ reduced bacteria viability, but none of the conditions they sued led to the complete biofilm eradication [65].

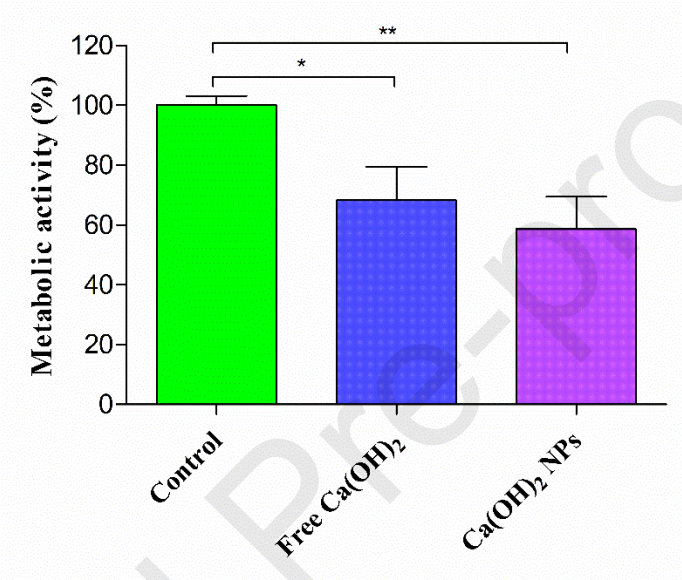


**Figure 6**. Metabolic activity of $Ca(OH)_2$ NPs and $Ca(OH)_2$ at 1 mg/mL using *Enterococcus faecalis* strain at 24 hours. 1x PBS was used as control. Statistical differences were calculated using one-way ANOVA and represented as *$p<0.05$ and **$p<0.01$.

Moreover, fluorescent images with *ex vivo* extracted human teeth were obtained staining viable bacteria using green fluorescence and dead ones using red label. These results confirm that both $Ca(OH)_2$ and $Ca(OH)_2$ NPs are able to decrease metabolic bacterial activity due to the release of hydroxyl ions at the therapeutic site of action. Despite the fact that no statistical differences were obtained on the metabolic activity against free $Ca(OH)_2$, $Ca(OH)_2$ NPs are able to decrease bacterial survival in a more effective manner than free $Ca(OH)_2$ and, at the same time, guarantee an increased internalization on the dentinal tubules as well as prolonged calcium hydroxide release [39]. In the apical region, a greater amount of dead bacteria was observed after the use of NPs (Figure 7). Clinically this is the most important and critical area because it is the area where the bacteria have the greatest capacity to obtain nutrients, and the most difficult to disinfect because it is also the area furthest away from access to the canal system. Therefore, the higher penetration of $Ca(OH)_2$ NPs [39] guarantee a more effective disinfection specifically in this complex area.

In addition, previous research has examined the efficacy of nanoparticles against *E. Faecalis* by confocal microscopy. In this area, Keskin et al. [66] found that when Chitosan NPs were

applied against E. Faecalis biofilms, there were no appreciable differences when compared to NaOCL (6%) [66]. Other nanoparticles types, like silver, have also demonstrated to decrease *E. Faecalis* bacterial biofilms in LIVE/DEAD ® testing conducted under Confocal Laser Scanning Microscopy [67,68].

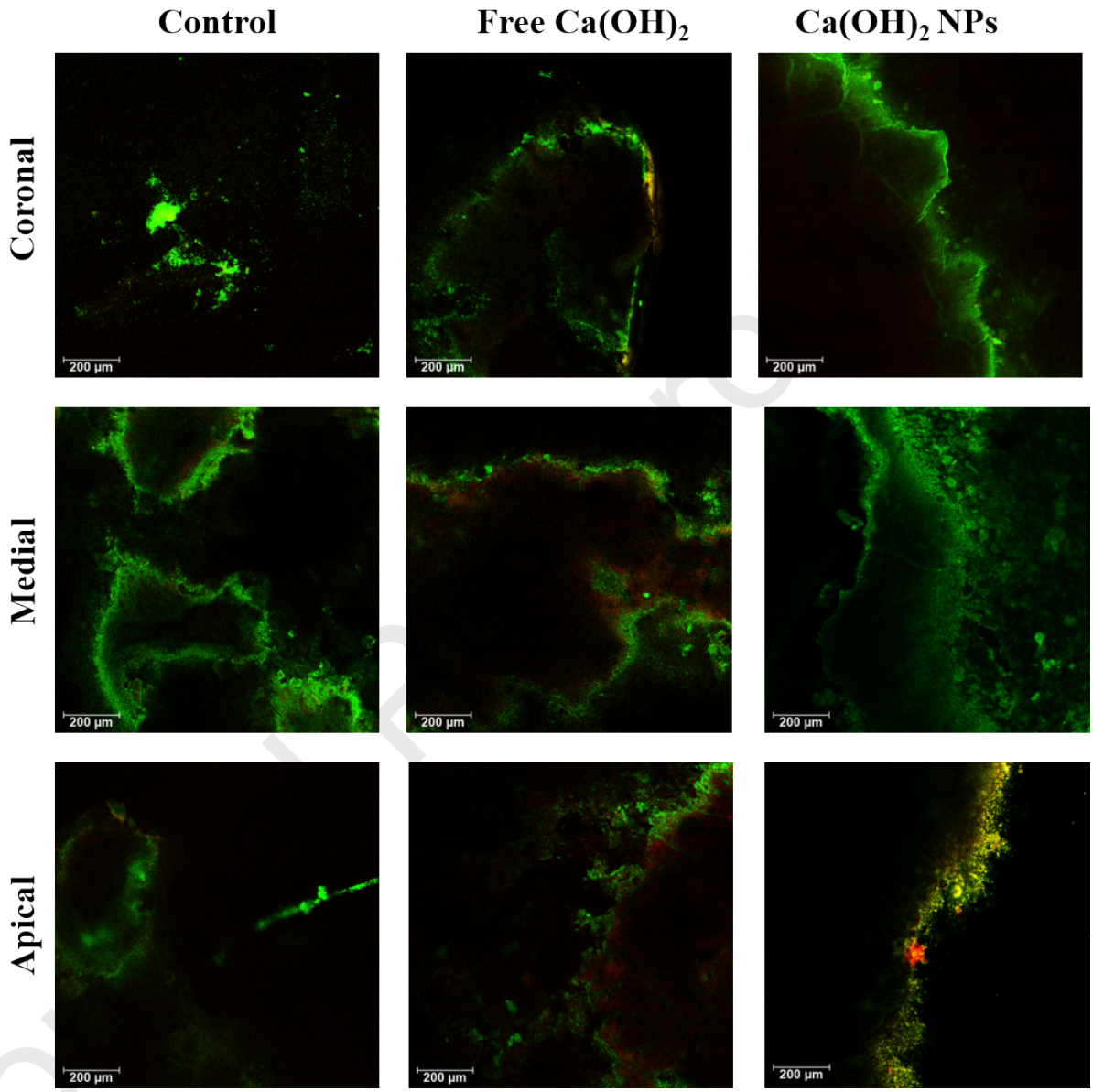


**Figure 7**. Metabolic activity of *Enterococcus faecalis* treated with $Ca(OH)_2$ NPs and calcium hydroxide at 1 mg/mL for 24 hours. Green bacteria are alive, red bacteria are dead and yellow bacteria, due to coalescence of live and dead staining, are cells that are metabolic actively but their membranes are compromised.

Although this results show the capacity of $Ca(OH)_2$ NPs to achieve suitable antibacterial capacity, the design of $Ca(OH)_2$ NPs may be further improved to increase its antibacterial properties either by increasing the $Ca(OH)_2$ concentration within the NPs or by combining it

with other antibacterial agents such as antibiotics or ions [64]. Moreover, the use of $Ca(OH)_2$ has been suggested to denature the collagen matrix or breakdown of the inorganic matrix of dentine [14]. This potential detrimental effect should be quantified in future experiments.

The null hypothesis has been rejected, and we found significant differences in antibacterial efficacy between the nanoparticle group and the control group. The minimum inhibitory concentration (MIC) in the nanoparticle group was higher than that in the commercial $Ca(OH)_2$ group. Regarding metabolic activity, there were no significant differences between the commercial $Ca(OH)_2$ group and the nanoparticle group, but there were differences compared to the control group.

## 4. Conclusions

Calcium hydroxide ($Ca(OH)_2$) has been considered the gold standard antibacterial agent in endodontics. However, innovative approaches have been explored to address limitations such as decreased antibacterial activity due to the buffering effect of dentin and difficulties accessing intricate root canal networks. In this area, nanotechnology-based medications offer potential solutions for these challenges.

This study shows that $Ca(OH)_2$ NPs possess an average size below 200 nm and exhibited a minimum inhibitory concentration (MIC) of 10 µg/mL for *Porphyromonas gingivalis* and *Enterococcus faecalis*, and 5 µg/mL for *Fusobacterium nucleatum*. Although no inhibition area was observed in the agar diffusion test, probably due to the agar buffering effect, $Ca(OH)_2$ NPs significantly reduced bacterial metabolic activity, thus preserving the active compound effectiveness.

To conclude, our findings highlight the potential of $Ca(OH)_2$ NPs as an antibacterial agent against several bacterial strains involved in endodontic infections being able to attain anatomically complicated infected areas such as the apical region. However, further testing, particularly against more resilient bacterial biofilms, is required before their clinical application can be carried out.

**Author Contributions:** F.M and MT.T were involved in performing all the antibacterial tests and experiments. All the antibacterial tests were done with the direct help and assistance of LM.D and R.P. Moreover, both JA.G and E.S.L were directly responsible for developing and designing the methodology of the study. Finally, L.M.D, K.I.A, ML.G, E.S.L and FD.S were involved in supervision of the writing process plus, article correction and editing.

**Data Availability Statement:** Data described in this paper are available on request.

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**Conflicts of Interest:** Dr. Firas Elmsmari, Dr. Fernando Duran-Sindreu, Dr. Marisa García López Dr. Jose Antonio González Sánchez and Dr Elena Sánchez López, report a licensed patent with the Universitat Internacional De Catalunya and University of Barcelona—Composition comprising nanoparticles, method for the preparation of a composition comprising nanoparticles and uses of the composition for dental treatment. European patent with the file number: EP20382504.

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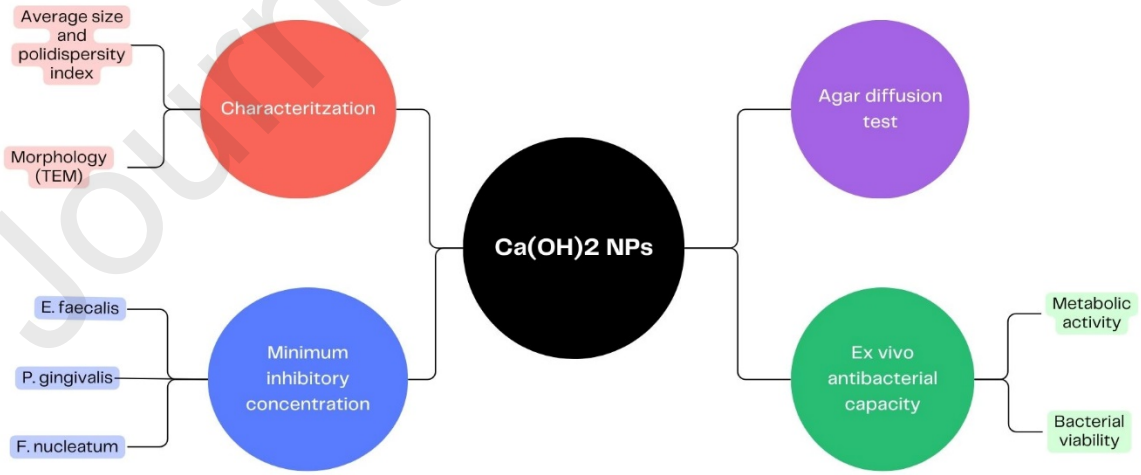

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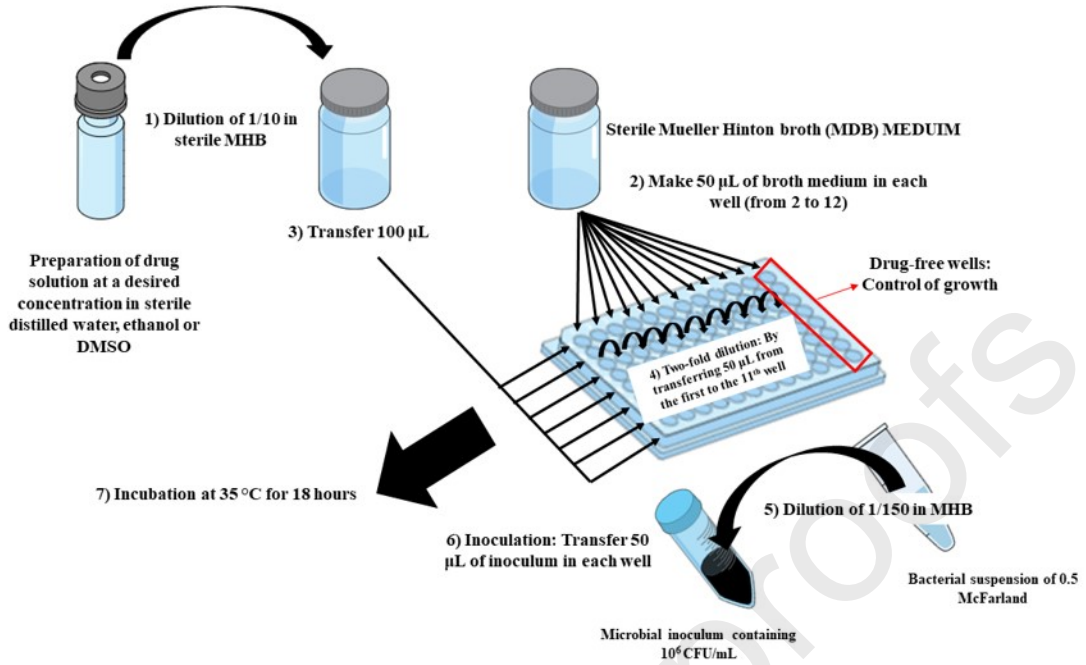


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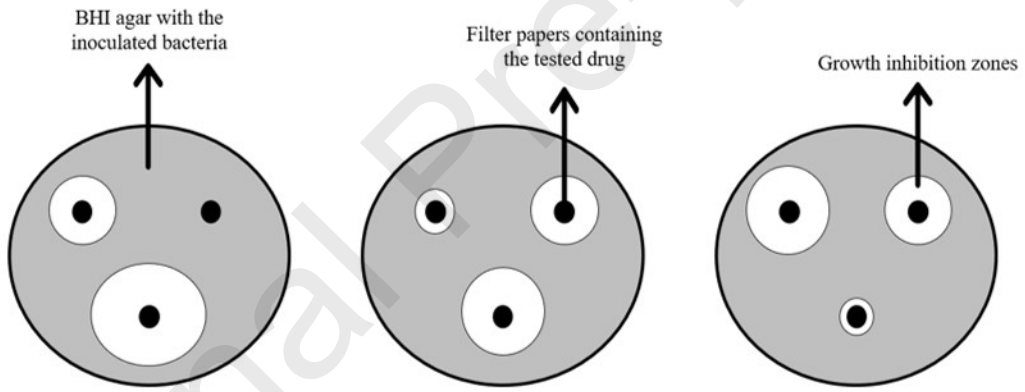

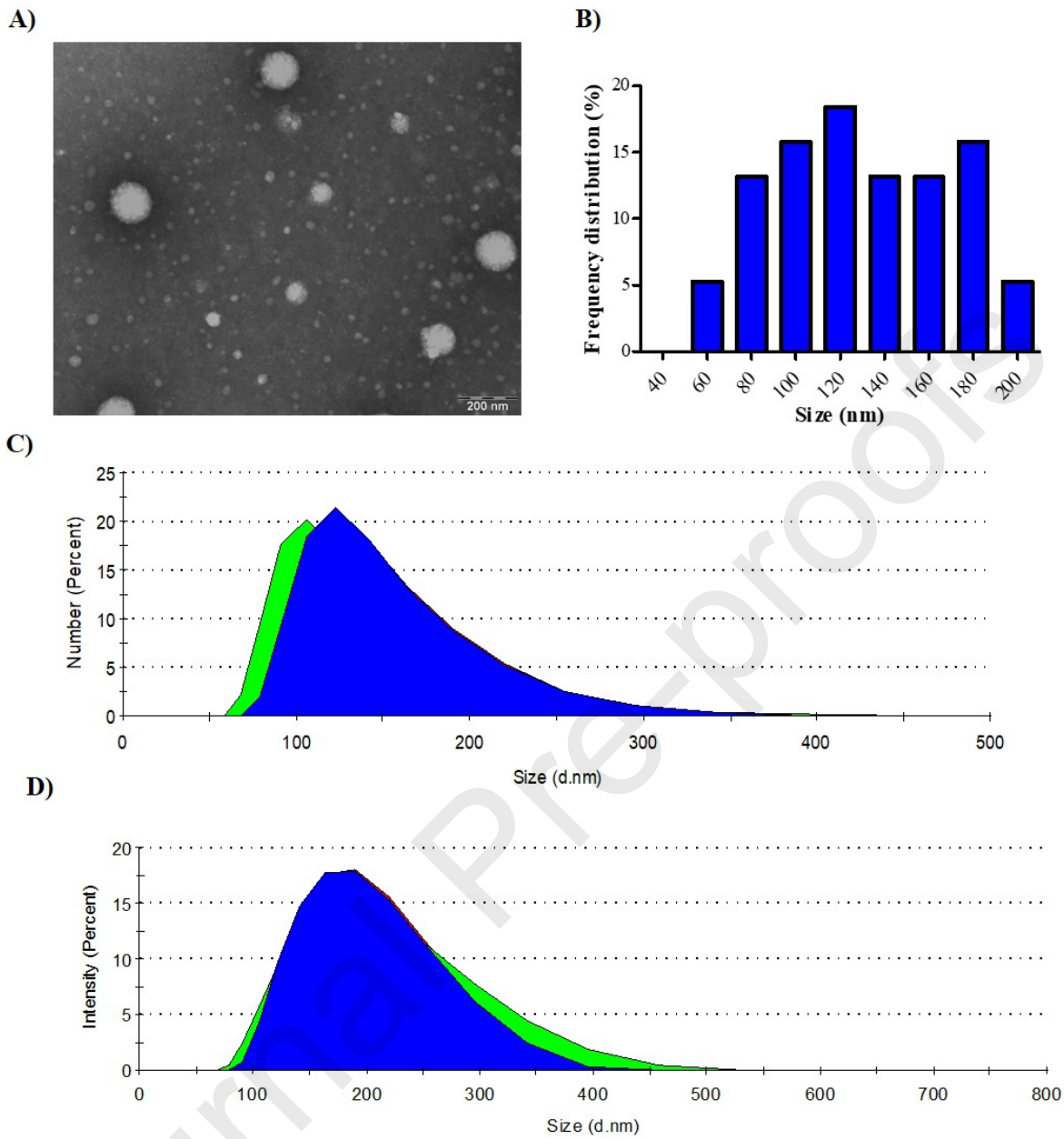

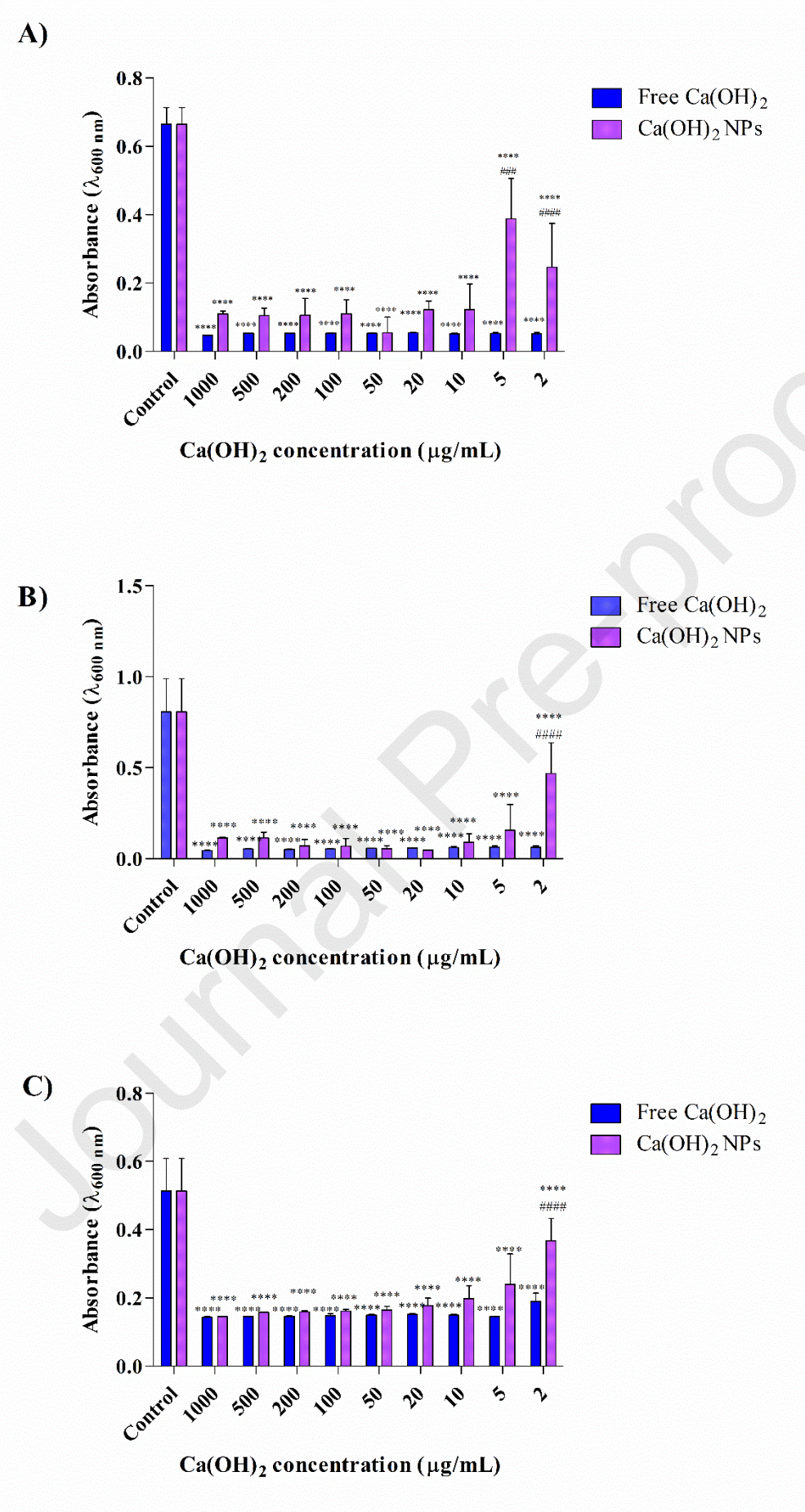

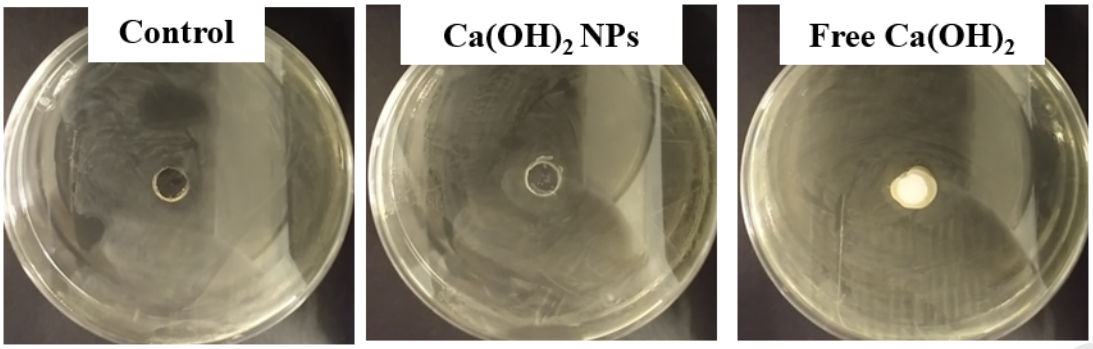


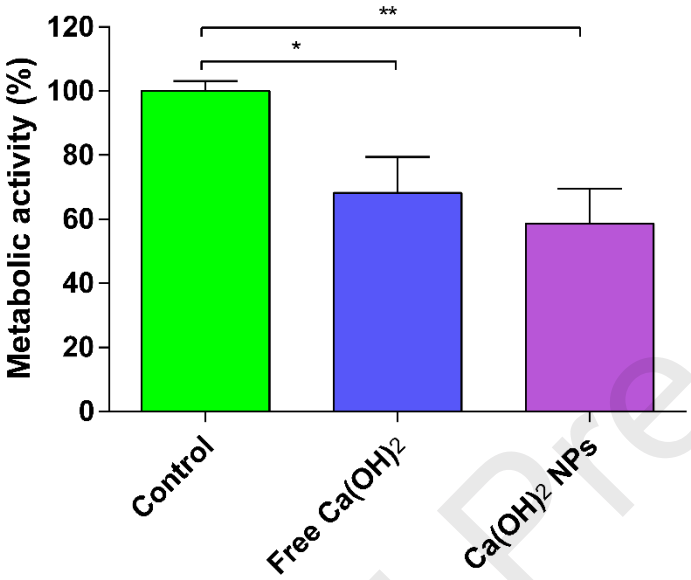

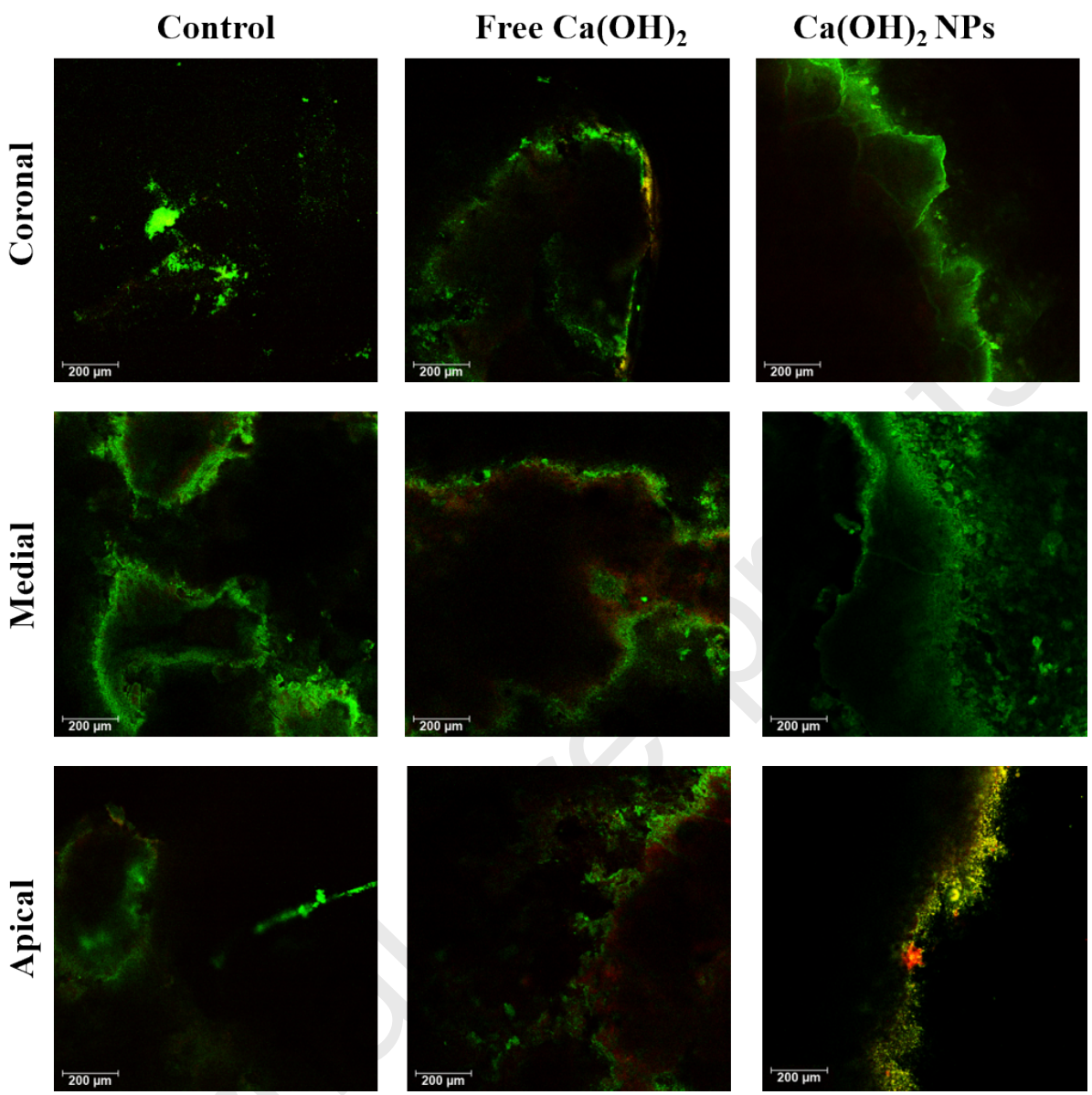


**Declaration of interests**

☐ 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.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Elena Sanchez-Lopez has patent #Composition comprising nanoparticles, method for the preparation of a composition comprising nanoparticles and uses of the composition for dental treatment. EP20382504.7 issued to Explotation rights: Universitat Internacional de Catalunya and Universitat de Barcelona. Regarding the patent mentioned other inventors are also included in this manuscript: García López, M. L.; Sánchez López, E.; Durán-Sindreu, F.; González Sánchez, J. A. and Elmasmari, F.

**Author Contributions:** F.M and MT.T were involved in performing all the antibacterial tests and experiments. All the antibacterial tests were done with the direct help and assistance of LM.D and R.P. Moreover, both JA.G and E.S.L were directly responsible for developing and designing the methodology of the study. Finally, L.M.D, K.I.A, ML.G, E.S.L and FD.S were involved in supervision of the writing process plus, article correction and editing.